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Incorporation of Adenine-8-C-14 and Orotic-6-C-14 Acid into Nucleic Acids of the Feline Neuraxis.* (23708)

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Present knowledge of nucleic acid metabolism in the mammalian neural axis is derived principally from histological studies and from the application of ultraviolet microspectroscopy to tissue sections(1-3). The glial elements have been generally neglected, nerve cell bodies being the chief object of study. Turnover studies involving orthophosphate-P-32(4-6) have revealed incorporation into nucleic acid phosphate in whole mammalian brain, but, according to Strickland(7), the values reported earlier are high because of contamination of the nucleic acid fraction by other phosphate-containing compounds of high specific activity. This author isolated the nucleic acids from the brains of cats given phosphate-P-32 intracisternally and sepa-

rated them into pentose nucleic acid (PNA) and deoxypentose nucleic acid (DNA) by alkaline hydrolysis. He observed appreciable incorporation of P-32 into PNA, but only slight incorporation into DNA. In subsequent papers from Rossiter's laboratory(8,9), it was demonstrated that an oxidative phosphorylating mechanism was involved in the incorporation of phosphate-P-32 into the PNA of cat brain slices *in vitro*. Bendich *et al.*(10) reported slight incorporation of formate-C-14 into brain PNA as compared with other viscera. However, they did not consider the question of the availability of the labeled precursor to the brain, *i.e.*, the blood-brain barrier. This communication presents the biochemical results obtained with the use of a labeled purine and a labeled pyrimidine precursor administered intrathecally in a series of adult cats. Avoidance of the blood-brain barrier by injection of the

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precursor directly into the cerebrospinal fluid permitted attainment of adequate levels of radioactivity with minimal doses of these expensive chemicals.

Methods. Adenine-8-C-14 (15.4×10^6 counts/min./mg) and orotic-6-C-14 acid (7.3×10^6 counts/min./mg), purine and pyrimidine precursors respectively, were injected intracisternally and in some instances into the lumbar subarachnoid space following laminectomy in cats anesthetized with sodium pentobarbital. Animals were killed under anesthesia by exsanguination at various time intervals following injection. The brain and cervical spinal cord with attached nerve roots and ganglia (also the lumbosacral spinal cord and radicular structures when intraspinal injection was performed) were removed and after appropriate tissue blocks were removed and placed in neutral 10% formalin for autoradiography, the remainder of the neural axis was preserved in a deep freeze at -10° to -20° until the biochemical studies could be carried out. The autoradiographic study is being reported in a separate communication. The spinal cord segments were maintained in the frozen state on ice while they were carefully dissected into gray and white matter for separate analysis; in some experiments spinal nerve roots and ganglia were also separated for analysis. A portion of the hindbrain was employed without further dissection. The tissue samples were weighed on a torsion balance and homogenized in 5 volumes of 10% NaCl (1 ml was the minimum volume used although samples of the ganglia and nerve roots frequently weighed less than 100 mg). The nucleic acids were extracted by boiling the homogenate in a water bath for one hour, removing the supernatant solution and boiling the residue in $2\frac{1}{2}$ volume of 10% NaCl for $\frac{1}{2}$ to 1 hour longer.[†] The combined NaCl solutions were chilled and mixed with 3 volumes of cold 95% ethanol. The precipitated sodium nucleates were removed by centrifugation, washed with cold 95% ethanol and with .05 N HCl, dried and incubated in 1-2 ml of .1N NaOH for 18-20 hours at 37°C . The DNA was precipitated by making the alkaline digest .1N acid with HCl, the PNA

remaining in the supernatant solution in the form of its constituent mononucleotides. The DNA precipitate was thoroughly washed in .05N HCl, dissolved in .1N NaOH, reprecipitated with 1N HCl to remove contaminating traces of ribonucleotides and dissolved in .02N NaOH. Further washing and reprecipitation did not alter the specific activity of DNA.[‡] Aliquots of the separated nucleic acid solutions were plated in duplicate on aluminum planchets for measurement of radioactivity; an aliquot was delivered from the same pipette into a test tube, and after dilution the absorbance at $260\text{ m}\mu$ was measured in a Beckman spectrophotometer in order to estimate the amount of nucleic acid plated.[§] The amount of PNA plated for counting

[†] Many authors have employed 10% NaCl for extraction of nucleic acids from tissues and alkaline hydrolysis for separation of PNA and DNA. Our procedure is the same as that of Hurlbert and Potter (11) with the exception that fresh frozen tissues were routinely used instead of acid- and lipid-extracted tissues. Control experiments revealed that omission of preliminary extraction in cold acid and in hot ethanol-ether did not alter the specific activity of isolated nucleic acids and that the specific activity remained constant in nucleic acids derived from successive extractions of tissues in boiling salt solution.

[‡] Analysis of several samples of brain DNA for pentose revealed the presence of 2-3.5% PNA. This is in agreement with Hurlbert and Potter (11) who found their DNA fraction contained less than 5% PNA. Histochemical studies (15) employing fixed specimens from tissues analyzed in our report revealed that most of the residual radioactivity was extractable from tissue sections by ribonuclease. In one experiment (CA5), sections of formalin-fixed spinal cord and brain which had been subjected to digestion by ribonuclease until no further radioactivity could be extracted lost a significant amount of radioactivity upon incubation in crystalline deoxyribonuclease solution. These experiments provide qualitative evidence for the presence of radioactivity in PNA and DNA.

[§] The amounts of PNA and DNA obtained depended upon the concentration of nucleic acids in the neural tissues studied and size of available samples. From 100-750 μg of each nucleic acid was extracted from spinal white and gray matter, 10-100 μg from spinal ganglia and roots and 1000-5000 μg from hindbrain.

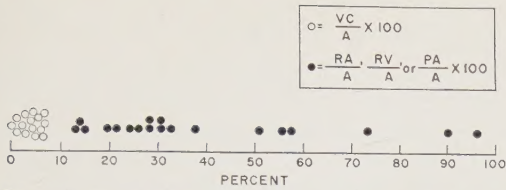


FIG. 3. The 10 sec. vena caval, right heart, and pulmonary artery krypton⁸⁵ concentrations expressed in % of the corresponding arterial concentrations. The vena caval samples were all proximal to the shunts while the right atrial, right ventricular, and pulmonary arterial samples were all distal to the shunts. A ratio of 10% divides the positive from the negative tests.

The Kr⁸⁵ content of pulmonary arterial blood was intermediate between systemic arterial and vena caval samples and reflected the presence of the left-to-right shunt. However, the 5 second pulmonary arterial samples often contained more radioactivity than the systemic arterial samples. This was probably attributable to the more rapid circulation between left atrium and pulmonary artery than between left atrium and a systemic artery. Desaturation was 90 to 95% complete in 5 minutes.

The arterial samples, after 10 seconds of inhalation, contained from 300 to 3800 counts per minute after the blank values of 30 to 100 counts per minute had been subtracted. The levels depended upon the gas concentration and depth of respiration. However, the arterial level was used only in relation to the right heart or vena caval level, and its absolute value was unimportant. The contents of the right heart and vena caval samples were thus expressed in relation to the simultaneous arterial content (Fig. 3). In all 15 studies, the vena caval samples, proximal to the shunts, were less than 8% of the arterial samples. The right atrial, right ventricular, and pulmonary arterial samples, distal to the shunts, all exceeded 12%. The 3 dogs with concentrations between 10 and 20% of the arterial levels each had repeat tests with concentrations of 20 to 30%. All of the experimental animals were sacrificed and in each the presence of a patent atrial septal defect was confirmed.

Discussion. The superiority of an inert gas in the detection of left-to-right shunts has

been demonstrated in studies comparing the nitrous-oxide test and the method of oxygen differences(1). The inert gas concentrations are independent of the patient's metabolic state and samples proximal to the site of the shunt are unnecessary. A radioactive gas would appear to be superior to nitrous-oxide in several respects. The analysis is much simpler and the results are available within a few minutes. Levels 10 to 100 times the blank value are easily achieved and the analytic error is thereby minimized. Thus, it is likely that diagnostic ratios may be established at far lower levels than with nitrous-oxide.

Kr⁸⁵ was selected in the present study because it was readily available and its applicability had been demonstrated in clinical studies of cerebral blood flow(2). The gas is stable and is rapidly excreted through the lungs. Since it is essentially a weak beta-ray emitter(3), it is relatively safe for laboratory personnel. Kr⁸⁵ is certainly not the only radioactive gas that can be used for the detection of left-to-right shunts. I¹³¹, as trifluoriodomethane, is being investigated in this laboratory and appears to give results similar to those obtained with Kr⁸⁵. A disadvantage of I¹³¹ is its half-life of 8 days, and any gas containing it must be obtained at frequent intervals. On the other hand, a single supply of Kr⁸⁵, with a half-life of about 10 years, can be used virtually indefinitely.

With the low concentrations of Kr⁸⁵ employed in these studies, high counts in one minute were best obtained by extracting the gas from the blood. An advantage of I¹³¹, a gamma emitter with a more penetrating radiation, is that it can be counted in blood without extraction. However, for the same reason, it is somewhat more hazardous to personnel. Continuing investigations will determine which isotope and compound is most suitable.

Summary. A method is described for the use of tracer doses of krypton⁸⁵ in the localization of left-to-right cardiac shunts. Fifteen studies were performed in 10 dogs with atrial septal defects. As the gas was inhaled, blood samples were drawn simultaneously from a vena cava, a right heart chamber, and a sys-

temic artery. The samples were analyzed for their radioactivity. The diagnosis of a shunt could be made when the ratio of radioactivity in right heart or pulmonary artery samples to that in systemic artery samples exceeded 10%. The apparent advantages of the method and the possible usefulness of other radioactive gases are discussed.

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✓ Detection of Circulatory Shunts by Use of a Radioactive Gas.* (23630)

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The most widely used technic for detection of left-to-right circulatory shunts requires the demonstration of a sudden increase in oxygen content at or past the site of the shunt. The widely varying flow rates and oxygen contents of blood entering the right atrium from the superior vena cava, inferior vena cava, azygous vein, and coronary sinus sometimes make it difficult to estimate the mixed venous oxygen content before the site of the shunt is reached. The oxygen contents of the cavae, which constitute the major part of the venous return, may differ by as much as 4 vol. %, and usually have a difference of one to 1½ vol. %(1). The comparative flow rates in the cavae, usually taken as a 2:1 ratio, are merely an estimate in any patient. Attempts to circumvent these problems have been made by the introduction of a foreign substance into the circulation. Swan and Wood(2) injected dye into the right side of the circulation and recorded dye dilution curves with an earpiece oximeter. Use of a foreign gas, nitrous oxide, was suggested by Callaway† and has been utilized in a large series of patients by Morrow, Sanders and Braunwald (1). They were able to detect 41 out of 43 shunts by taking an integrated one minute sample simultaneously from an intracardiac

catheter and a peripheral artery during the inhalation of 15-25% nitrous oxide. However, the shunt was missed in 11 of these 43 patients by use of the oxygen "step-up" method. The superiority of the nitrous oxide method is evident, but there are disadvantages. Analyses of nitrous oxide samples are difficult and time consuming. A high blank value is always present, and it may sometimes be difficult to achieve a significant arterial level. The use of an innocuous foreign gas which is easily absorbed and analyzed would simplify the situation. Dr. Louis Dotti of this hospital suggested a radioactive gas. This paper describes our experiences with ethyl iodide, containing radioactive I¹³¹. This gas (without the I¹³¹) was used extensively 32 years ago in human cardiac output determination(3) and was proved to be without ill effects. Analysis of blood containing radioactive ethyl iodide is achieved simply by placing a one half or one cc sample in a well counter for one or 2 minutes. The I¹³¹ half life of 8 days provides a safety factor and facilitates easy handling.

Method. Dogs weighing from 10 to 20 kg were anesthetized with pentobarbital, and a Cournand catheter was inserted (under fluoroscopic guidance) into the pulmonary artery via the jugular vein. This position was confirmed by pressure traces before and after each ethyl iodide administration and by withdrawal pressure tracings. Catheters were also

* This work was subsidized by N. Y. Heart Assn. and Natl. Heart Inst., N.I.H.

† Unpublished data.

inserted (under fluoroscopy) into the superior or inferior vena cava and into the femoral artery. Each catheter and stop-cock system contained a dead space of 1.5 cc. An endotracheal tube with an inflatable cuff was inserted into the trachea. The ampule containing the radioactive gas was placed in a piece of polyvinyl tubing and both ends clamped. This was connected to a one-way respiratory valve attached to the endotracheal tube. The ampule was crushed with a hammer and the clamps removed. Exhaust from the valve was collected in a large polyvinyl bag, or a series of bottles containing ethyl alcohol which absorbs the radioactive gas. A by-pass allowed the animal to breathe until the clamps were removed from the tubing containing the radioactive gas. Blood samples were taken simultaneously from the pulmonary artery, femoral artery, and vena cava at approximately 5 second intervals by withdrawing 1.5 cc into a syringe through a 3-way stop cock and ejecting into test tubes containing heparin as an anticoagulant. One or one half cc samples were pipetted from the collection tubes into lusteroid counting tubes, and radioactive content was measured in a standard well counter with a scintillation detector. Atrial septal defects were prepared by right thoracotomy, venous inflow occlusion, and removal of a piece of interatrial septum 7 to 20 mm in diameter under direct vision. Circulation was restored, the atrium sutured, and the chest closed. Ethyl iodide was then administered, or in some cases, was given after an interval of several weeks. Radioactive ethyl iodide containing I^{131} was obtained from the Radiochemical Centre, Amersham, England. It contains a specific activity of 5 millicuries per mmole. Ampules used contained 11 to 137 microcuries.

Results. Ethyl iodide was administered 19 different times. Five of these experiments were considered to be technically unsatisfactory and are excluded from the data. There are 8 studies in normal dogs and 6 on dogs with atrial septal defects. An additional element of control was present in that 4 of the 6 atrial defect dogs had ethyl iodide studies prior to the preparation of the defect.

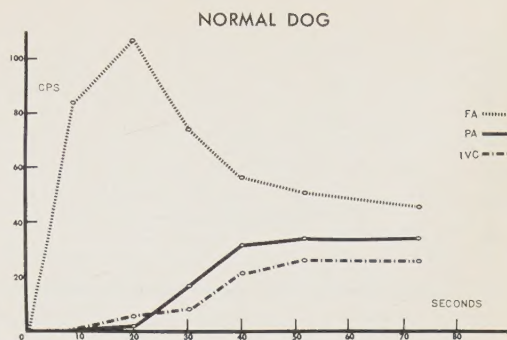


FIG. 1. CPS represents radioactivity in counts per sec. FA, PA, and IVC represent radioactivity in femoral artery, pulmonary artery, and inferior vena cava, respectively.

Normal dogs. Results in a typical normal dog are seen in Fig. 1, where the radioactivity in counts per second is plotted against the time of sampling. Radioactivity in femoral arterial samples is seen to rise rapidly to a high value and then to fall off toward an equilibrium value. Measurable concentrations appear in the pulmonary artery at 20 seconds and continue to rise as arterial values fall. The concentration in the inferior vena cava is initially higher than in the pulmonary artery, but then falls below it. Samples were also taken up to several hours, but equilibrium was reached in 5 to 8 minutes with all 3 values identical. They fell off slowly thereafter. It should be noted that significant levels were easily reached in this 15 kg dog using the low dose of 35 microcuries.

Although femoral artery and pulmonary artery curves followed the same pattern in all normal dogs, venous curves varied depending on the sampling site. Inferior vena cava values were at times slightly higher than pulmonary artery values for the first 25 seconds. Inferior vena cava samples were usually higher than superior vena cava or femoral vein samples.

Atrial defect dogs. Fig. 2 shows the results of administering 32 microcuries of ethyl iodide to a 20 kg dog in which an atrial septal defect had been prepared 4 months previously. The pulmonary artery radioactivity is at a high level in the first sample and remains high throughout the sampling period. Inferior vena cava values are similar to those in the

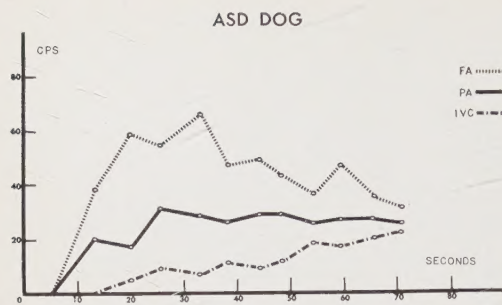


FIG. 2. Symbols similar to those in Fig. 1.

normal dog of Fig. 1. Autopsy of this dog demonstrated a well healed atrial septal defect 12 mm in diameter. The rapid rise of pulmonary arterial values is clear evidence of a left-to-right shunt. In 2 of the atrial septal defect dogs, the first pulmonary artery sample is higher than the corresponding arterial sample. This is to be expected if the samples are taken early enough following the inhalation of ethyl iodide.

In all dogs with an acute or chronic atrial septal defect, the results were similar to those in Fig. 2 during the first 20 seconds. However, the caval sample after 20 seconds was sometimes seen to rise above the pulmonary arterial level, especially where arterial levels were seen to fall off rapidly after a high initial value. Presumably recirculation causes the high venous level.

In the shunt dogs, pulmonary artery values usually fell with a rapidly falling arterial content, whereas in the normals, pulmonary artery values continued to rise.

In 2 dogs, catheters were placed in the pulmonary artery, femoral artery, and the vena cava as described above. Normal studies were performed, an atrial defect was made, the chest closed, and a second ampule of ethyl iodide was administered. The first ampule gave typically normal results. Results from the second ampule were similar to those of the atrial defect dogs, with the addition of a high base line.

Comparison of ASD and normal dogs. The best indication of a left-to-right shunt seems to appear in the early part of the curves before recirculation plays an important part in the pulmonary arterial concentration of radioactivity. Comparison of pulmonary artery

and arterial concentration at identical times after administration of ethyl iodide would be the best indication of the presence of a shunt. However, the intermittent sampling technic and the fact that ethyl iodide was inhaled rather than injected does not allow measurement of the exact time radioactivity reaches the pulmonary veins. Therefore, the time chosen for comparison was at the highest arterial level reached within the first 14 seconds after radioactivity was first noticed in the arterial blood. This eliminated the problem of recirculation and also the complication of a falling arterial level.

Table I presents results of the comparison of pulmonary artery and femoral artery values at the times indicated. This shows in the normals a range of 0% to 4.9%, with an average value of 1.6%, whereas the atrial defect dogs had a range of 20.0% to 60.6%, with an average of 43.9%.

In addition, samples compared at any time earlier than that shown in Table I always showed an even lower pulmonary artery to femoral artery ratio. Earlier comparison in the atrial defect dogs nearly always showed a higher ratio than that indicated in Table I, with pulmonary artery sometimes exceeding femoral artery.

The metabolism of ethyl iodide is being studied, but indications are that radioactivity

TABLE I.* Radioactivity in Pulmonary and Femoral Arteries during Inhalation of Radioactive Ethyl Iodide in Dogs.

| | Time after 1st arterial radioac- tivity, sec. | PA (counts/sec.) | FA | PA/FA, % |
|-------------------------|--|---------------------|------|-------------|
| Normal | 10 | 1.5 | 107 | 1.4 |
| | 12 | 0 | 153 | 0 |
| | 15 | 0 | 121 | 0 |
| | 7 | 0 | 7.5 | 0 |
| | 6 | 15 | 305 | 4.9 |
| | 11 | 0 | 45 | 0 |
| | 10 | 9 | 185 | 4.8 |
| | Avg | | | 1.6 |
| Atrial septal defect | 13 | 31 | 55 | 56.4 |
| | 8 | 7 | 18.8 | 37.2 |
| | 12 | 53 | 92 | 57.6 |
| | 13 | 40 | 200 | 20 |
| | 11 | 97 | 160 | 60.6 |
| | 9 | 39 | 152 | 25.7 |
| | Avg | | | 42.9 |

* Background counts averaged 3.9 counts/sec.

is rapidly excreted in the urine and expired air. Studies on the uptake of the thyroid gland are also in progress.

Discussion. Use of the radioactive gas as described gives a clear indication of the presence of an atrial septal defect and the technic presumably will also apply in ventricular septal defect, anomalous pulmonary venous drainage, patent ductus arteriosus, and other types of left-to-right shunts. In view of the low amount of radioactivity, and lack of toxicity of ethyl iodide, the method would seem applicable in human diagnostic procedures. It would have the added feature of rapid, accurate analysis, so that the presence of a shunt might be known within a few minutes, while the catheter is still in position. Additional ampules may be administered for more exact localization of the defect.

It would also seem possible to calculate the amount of left-to-right shunt by a simple mixing formula.

The ratio of shunt flow to pulmonary flow should be determined by the following formula:

$$\frac{Q_s}{Q_{pf}} = \frac{C_{pa} - C_{cav}}{C_a - C_{cav}}$$

Where: Q_s —amount of shunt flow
 Q_{pf} —amount of pulmonary flow
 C_{pa} —conc. of radioactivity in the pulmonary artery
 C_{cav} —conc. of radioactivity in the cavae
 C_a —conc. of radioactivity in the femoral artery

The validity of the above formula is now under investigation. It may be possible to use values before the onset of recirculation, in which case concentration in the cavae will not be a factor. The use of mean values as ob-

tained in an integrated sample, or by use of the area under the curves will be necessary. Measurement of coronary flow simply by pulmonary arterial catheterization may be possible by use of these formulae and a gas which is not absorbed by the tissue. Coronary sinus blood would then have the same concentration as arterial blood. A radioactive gas, krypton 85, has been used by Hansen(4) to replace nitrous oxide when measuring coronary flow by the method of Kety and Schmidt (5) using coronary sinus catheterization. Krypton 85 has a half life of 9.4 years, and it would seem that ethyl iodide with a half life of 8 days would be safer and more convenient for this purpose.

The possibility of using this gas for pulmonary diffusion problems should also be considered.

Summary. A radioactive gas, ethyl iodide, containing I^{131} , has been administered to normal dogs and dogs with atrial septal defects. Simultaneous samples taken in the pulmonary artery and femoral artery give a clear indication of the presence of the shunt. Its use in human diagnostic procedures is suggested.

The authors are indebted to Dr. Daniel Lukas for his criticisms and suggestions.

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Pancreatic Elastase I. Observations on Cellular Source and Endocrine Influence. (23631)

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Elastase, a pancreatic enzyme characterized by its ability to dissolve elastin, has been isolated and crystallized by Balo and Banga(1). Its physico-chemical and enzymatic properties have been further investigated by Lewis *et al.*(2). Concerning its pancreatic origin, Lansing(3) has claimed that elastase is secreted by the α -cells of the pancreas and this concept has been furthered by Carter(4) who found that cobalt treatment caused a depletion of the elastase content of the dog pancreas. These conclusions are not consistent with the data of Kokas(5) and Lewis, *et al.*(2) who have demonstrated the presence of elastase in the pancreatic juice of the dog. We became interested in elastase as a result of our studies dealing with the possible anti-atherogenic effect of the enzyme. During the course of these studies, which included endocrine as well as other forms of experimental manipulation, changes in the elastase content of the pancreas were noted. These studies were then expanded to include parallel measurements of the pancreatic content of one of the known digestive enzymes, *i.e.* lipase, along with elastase.

Material and methods. Male rats of the Sprague-Dawley strain (120-150 g) and male guinea pigs (500 g) were used in our experiments. Upon sacrificing the animals, the pancreas was completely removed, weighed and extracted by homogenizing in a Waring blender with a measured amount of chilled distilled water so that the final extract contained the equivalent of 5 mg pancreas/ml. Elastase was determined by a modification of the method of Hall(6). Fifty milligrams elastin (prepared from horse ligamentum nuchae) was suspended in 10 ml 0.1 M glycine buffer pH 8.9 and was incubated with the tissue extract for 18 hr at 37°C. At the end of this period, the digest was filtered and the amount of solubilized elastin determined by means of the biuret reaction. A linear re-

sponse is obtained with both crystalline elastase and pancreatic extracts. The elastase units are expressed in terms of the reading obtained with the Klett colorimeter using a 515 m μ filter. Lipase was measured by a phototurbidometric method, using a commercially available coconut oil emulsion.* The substrate was prepared by addition of 0.2 ml of the oil emulsion to 100 ml of a 0.2 M phosphate buffer, pH 8.1, containing 0.1% sodium desoxycholate (w/v). The substrate was brought to, and maintained at, 37°C in a constant temperature bath. One milliliter of the enzyme solution was added to 4.0 ml of the warmed substrate and the time in seconds for a 50% reduction in optical density was recorded. The enzyme-substrate solution was kept at 37°C between readings. A "Spectronic 20" colorimeter (Bausch and Lomb Co.), at a wavelength of 600 m μ , was used for all determinations. The colorimeter was arbitrarily set at an optical density reading of 0.8 at 0 time by the substitution of 1.0 ml of distilled water for the enzyme solution. The specificity of the substrate for determining pancreatic lipase can further be demonstrated from the data in Table I. Of all the tissues studied, only the pancreatic homogenate caused a rapid clearing of substrate in relatively low tissue concentrations. When the time in seconds for a 50% reduction in optical density is plotted against lipase concentration using logarithmic coordinates, a straight line is obtained.

Results. To determine whether food intake would be a critical factor in evaluating changes in the pancreatic enzymes, a group of animals was starved and the enzyme concentration of the pancreas was compared with that of animals fed *ad lib*. The results (Tables II and III) indicate a marked increase in the enzyme content of the pancreas of both the starved rat and guinea pig. This

* Ediol, Schenley Lab., Inc.

TABLE I. Reduction in Optical Density of a Coconut Oil Substrate for Various Rat Tissue Homogenates.

| Tissue | No. of determinations | Homogenate, conc./ml* (wet wt) | Time for 50% reduction in optical density | |
|----------|-----------------------|--------------------------------|---|----------------|
| | | | Min. | Sec. |
| Pancreas | 7 | 5 mg | | 120 \pm 9.9 |
| " | 7 | 2½ mg | | 211 \pm 15.4 |
| " | 7 | 1¼ mg | | 370 \pm 27.8 |
| Spleen | 7 | 5 mg | 95 \pm 8.6 | |
| Serum | 4 | 1 ml | 150† | |
| Adrenal | 7 | 5 mg | 150† | |
| Heart | 7 | | | |
| Stomach | 3 | | | |
| Liver | 7 | | | |
| Muscle | 3 | | | |
| Marrow | 3 | | | |
| Lung | 3 | | | |

* One ml of tissue homogenate added to 4.0 ml of substrate and the time for a 50% reduction in optical density recorded.

† No appreciable substrate clearing observed at this time.

increase in elastase parallels that of lipase both in direction and degree. These data demonstrated the necessity for paired feeding in all subsequent experiments.

Further reference to the data in Tables II and III shows that any experimental procedure which causes an increase or decrease in the elastase concentration of the pancreas results in a similar change in the lipase activity of the organ. This is readily apparent when the ratio exp./control for each enzyme is calculated. Pilocarpine markedly depletes

pancreatic elastase and lipase, and causes the appearance of these enzymes in the urine. The duodenal contents were found to contain elastase before and after pilocarpine administration. A decreased concentration of the elastase and lipase content of the pancreas was found following hypophysectomy (Table II).

The guinea pig responds to cobalt administration by depletion of both the elastase and lipase content of the pancreas, whereas the rat—even at near lethal cobalt levels—shows no change. Administration of thiouracil to rats (Table II) caused a marked reduction of the lipase and elastase concentration of the pancreas.

Discussion. Lansing(3) has stated that the pancreatic alpha-cell is the source of elastase inasmuch as he could extract it from the islet tissue of the teleost fish, the pancreas of which is divided into distinct islet and acinar zones. Carter(4), using cobalt to destroy the alpha-cells of the dog pancreas, found a marked reduction in the elastase content of this organ, and interpreted this data as confirmation of Lansing's claim(3). Our data indicate that elastase, at least in the rat and guinea pig, is a digestive enzyme secreted by the acinar tissue of the pancreas. This is inferred from the findings that (1) any experimental procedure which causes a change in elastase concentration also elicits a similar

TABLE II. Changes in Pancreatic Elastase and Lipase in the Rat.

| Treatment | Pancreatic wt, mg/100 g B.W. | | Elastase, units/mg pancreas | | Elastase, exp./control | Lipase (time in sec. for 50% lipolysis/2.5 mg pancreas) | | Lipase, exp./control¶ |
|-------------------|------------------------------|----------|-----------------------------|-------------|------------------------|---|----------|-----------------------|
| Starvation*(5) | 250 \pm 22 | N.S. | 105 \pm 9.2 | P = <.01 | 2.06 | 74 \pm 31.6 | P = <.05 | 2.34 |
| Control (5) | 300 \pm 17 | | 51 \pm 12.6 | | | 166 \pm 6.0 | | |
| Pilocarpine†(5) | 369 \pm 62 | N.S. | 33 \pm 3.8 | <i>Idem</i> | .39 | 463 \pm 62.7 | P = <.01 | .31 |
| Control (5) | 389 \pm 24 | | 84 \pm 3.9 | | | 151 \pm 10.3 | | |
| Hypox‡(10) | 258 \pm 21 | P = <.05 | 44 \pm 5.3 | " | .59 | 262 \pm 37.4 | P = <.05 | .54 |
| Control (6) | 343 \pm 21 | | 75 \pm 6.2 | | | 138 \pm 22.9 | | |
| Cobalt§(8) | 348 \pm 30 | N.S. | 98 \pm 20 | N.S. | — | 171 \pm 13.7 | N.S. | — |
| Control (8) | 322 \pm 17 | | 86 \pm 4.2 | | | 181 \pm 8.7 | | |
| Thiouracil (10) | 284 \pm 22 | N.S. | 17 \pm 2.6 | P = <.01 | .22 | 616 \pm 83.4 | P = <.01 | .29 |
| Control (10) | 316 \pm 22 | | 78 \pm 6.4 | | | 182 \pm 19.7 | | |

* 5-day starvation. † 4 mg pilocarpine S.C., pancreas removed 4 hr after inj. ‡ 4-wk hypox.
§ 7 mg cobalt chloride/7 day S.C. || Thiouracil, 0.2% in diet for 3 wk. ¶ Reciprocal ratio since an inverse relationship exists in the assay between activity and time of lipolysis.

No. in parentheses = No. of animals in group.

TABLE III. Changes in Pancreatic Elastase and Lipase in the Guinea Pig.

| Treatment | Pancreatic wt, mg/100 g B.W. | Elastase, units/12 mg pancreas | Elastase, exp./con- trol | Lipase (time in sec. for 50% lipoly- sis/12 mg pancreas) | Lipase, exp./con- trol |
|-----------------|------------------------------------|--------------------------------------|--------------------------------|--|------------------------------|
| Starvation* (6) | 242 \pm 25 | 73 \pm 11.3 | 1.55 | 228 \pm 22 | 1.54 |
| Cobalt† (8) | 213 \pm 18 | 20 \pm 1.4 | .42 | 1423 \pm 180 | .27 |
| Controls (10) | 241 \pm 19 | 47 \pm 7.4 | | 387 \pm 48 | |

* 5-day starvation.

† 25 mg cobalt chloride daily for 7 days.

No. in parentheses = No. of animals in group.

change in lipase concentration both in direction and degree. (2) Elastase is present in duodenal juice and is depleted from the pancreas by pilocarpine. (3) The observation that cobalt causes a depletion of the elastase and lipase content of guinea pig pancreas, whereas the rat pancreas is unaffected, is paralleled by the reports of other investigations (8,9) that the rat is resistant to the hypothyroid effect of cobalt; whereas the guinea pig is markedly sensitive to this activity. Accordingly, we postulated that perhaps the depletion of pancreatic elastase and lipase was the result of hypothyroidism or some mechanism which results in a lowered basal metabolic rate. This postulate was strengthened by the finding that thiouracil administration to the rat results in a depletion of the elastase and lipase content of the pancreas as is also observed after hypophysectomy. Shafer (10) has also implicated the thyroid in the maintenance of the normal appearance and proteolytic activity of the rat submaxillary gland. Perhaps Carter's (4) data may be reinterpreted in this fashion. (4) Our finding that hypophysectomy results in a decreased concentration of elastase and lipase of the rat pancreas, is in accord with the work of Baker (7) who reported a similar depletion of pancreatic proteinase after hypophysectomy. These data are difficult to reconcile with the concept that elastase is secreted by the alpha-cell of the pancreas inasmuch as Volk *et al.* (11) have found that in the rat, hypophysectomized for 4½ months, there is no change in alpha-cell cytology or occurrence.

Summary. 1. Concentration of elastase

and lipase in the pancreas of rat and guinea pig responds similarly to various experimental manipulations. The changes are parallel both in direction and degree. Starvation causes an increased concentration, whereas pilocarpine, hypophysectomy, thiouracil treatment result in a marked depletion of these pancreatic enzymes. Cobalt administration to the guinea pig causes a depletion of pancreatic elastase and lipase, whereas in the rat such an effect is not obtained. This is attributed to the relative resistance of the rat to the hypothyroid effect of cobalt. 2. Based on above observations, it is inferred that in the rat and guinea pig, elastase is a digestive enzyme elaborated by the acinar tissue of the pancreas.

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Transport of Epinephrine and Norepinephrine in Human Plasma.* (23632)

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(Introduced by G. W. Thorn)

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Plasma proteins may interact with other proteins, with ions, and with small molecules. The present communication concerns the transport of epinephrine (E) and norepinephrine (NE) in human plasma and their binding with plasma proteins.

Materials and methods. The blood employed was collected either in ACD[†] solution or through cationic exchange resins (Dowex-50) in the ADL-Cohn Blood Fractionator(1). The cold ethanol Method 6, described by Cohn and his collaborators, was employed for the fractionation of the plasma pools(2). The separated fractions (Table I) were dissolved or suspended in the smallest possible volume of 0.15 M sodium chloride and were dialyzed for a limited period of time (2 to 3 hours) against 0.15 M sodium chloride at a pH approximately 6.0, at 2°C. Chemical determinations for E and NE were performed with the method of Weil-Malherbe and Bone(3) as modified by Aronow(4). Bioassays for E and NE were performed by a modification of the method described by Gaddum and Lenbeck(5). The bioassay is based on the quantitative inhibition by the catechol amines of the contraction induced with acetylcholine in an *in vitro* preparation of rat colon and rat uterus. The concentration of the fractions, tested with the bioassay, over the original plasma volume was 8 to 10 fold.

Results. Two ACD and 2 resin-collected plasma pools were fractionated within 24 hours from the collection of the blood. The separated fractions, after a limited dialysis, were stored in the frozen state at -5°C.

* This work has been supported by Grants from N.I.H. and Dept. of Army, and by gifts from Kresge Fn., King Fn., and from industry.

[†] ACD solution was prepared according to NIH Formula A: 8 g citric acid • 1 H₂O; 22 g dextrose (anhydrous); 26 g sodium citrate • 2 H₂O; made up to 1 liter with distilled water. Five hundred ml of blood is collected into 75 ml of the above solution.

Table I shows distribution of E and NE among the plasma fractions. It is suggested, from the results, that the E is almost completely bound to human plasma proteins and only a trace is unbound (supernatant fluid V). The protein responsible for the major binding and the transport of E is the albumin, which is present in Fraction V. In one case Fraction IV-4, determined by the chemical method, also was found active. The NE is also bound to albumin of Fraction V; however, an appreciable amount of NE seems to be unbound (supernatant fluid V).

Fraction V was subjected to acid hydrolysis by boiling for 15 minutes at pH 1. The amount of E and especially NE after the acid hydrolysis was found significantly increased, as determined with the chemical assay. Duplicate determinations were used in these experiments. The amount of E before the acid hydrolysis was found to be .8 µg per liter of original plasma; after the acid hydrolysis it was 1.0 µg per liter of plasma. The amount of NE before the acid hydrolysis was 2.0 µg per liter of plasma and 8.0 µg after the acid hydrolysis. It is of interest to notice that the amount of NE of Fraction V determined after the acid hydrolysis by the chemical method is very close to the amount in the untreated Fraction V as determined by the bioassay method (9.4 µg/liter of plasma).

Summary. Plasma pools collected in ACD solution or through cationic exchange resins were fractionated with the cold ethanol Method 6 of plasma fractionation, and the separated fractions were analyzed for E and NE, chemically and biologically. Although the E is almost completely bound to plasma proteins, an appreciable amount of NE seems to be unbound. The plasma protein responsible for the binding and the transport of both E and NE is in the albumin. A part of E possibly binds also with Fraction IV-4. The increase of E and NE activity following acid

TABLE I. Distribution of Epinephrine and Norepinephrine in Human Plasma Fractions.

| Plasma fractions | Epinephrine ($\mu\text{g}/\text{l}$ of plasma) | | | Norepinephrine ($\mu\text{g}/\text{l}$ of plasma) | |
|---------------------|---|---------|---------------------|--|---------------------|
| | Chemical method Exp. 1* | Exp. 2† | Bioassay Exp. 3‡ | Chemical method Exp. 1§ | Bioassay Exp. 2‡ |
| Plasma control | 2.1 | | Incr. contr. | 5.4 | Incr. contr. |
| Precipitate I | .2 | .1 | Inactive | .1 | Inactive |
| II + III | .2 | .2 | " | .2 | " |
| IV-1 | .2 | .2 | " | .2 | " |
| IV-4 | .2 | .6 | " | .16 | " |
| V | .9 | 1.0 | .62 .8 | .85 | 9.4 2.0 |
| Supernatant fluid V | .16 | | Trace | 1.2 | 2.38 |

* Resin-collected plasma (6 donors).

† ACD- " " (10 ").

‡ Resin- " " (9 ").

§ ACD- " " (5 ") one wk old.

|| Determined by chemical method.

hydrolysis suggests the presence of a conjugated form, binding to albumin, which is released by acid hydrolysis.

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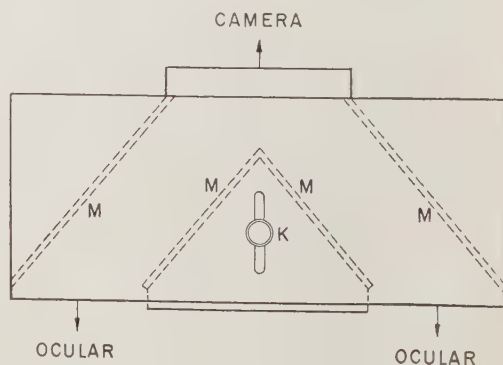
Device for Simultaneous Microscopic Observation of Peripheral and Visceral Circulation.* (23633)

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A method has been developed for simultaneous comparison and photographic recording of peripheral and visceral vascular areas in the same experimental animal.

Methods. The apparatus consists of a field-splitting device† (Fig. 1) which is placed over the oculars of 2 microscopes so adapted that the interocular distance is equal to the average distance between the cheek pouch and mesocecum of the hamster. The cheek pouch is pinned over a small transparent lucite block and prepared as a single membrane(1). The method for exposing and spanning the meso-



M = FRONT SURFACE MIRROR.

K = SET KNOB. CENTER PAIR OF MIRRORS MAY BE ADJUSTED UP OR DOWN.

FIG. 1. Field-splitting device.

* Aided by contract from Air Force Office of Scientific Research.

† Made to author's specifications by Mr. John B. Sanroma, Mass. Inst. Tech.



FIG. 2. Cheek pouch and mesoappendix preparation.

cecum is a modification of that introduced by Zweifach(2). Both vascular areas are manipulated by separate mechanical stages so that any adjustment of either field has no effect on the other (Fig. 2). Continuous temperature measurements of a thermoregulated mammalian Ringer irrigating solution are made by

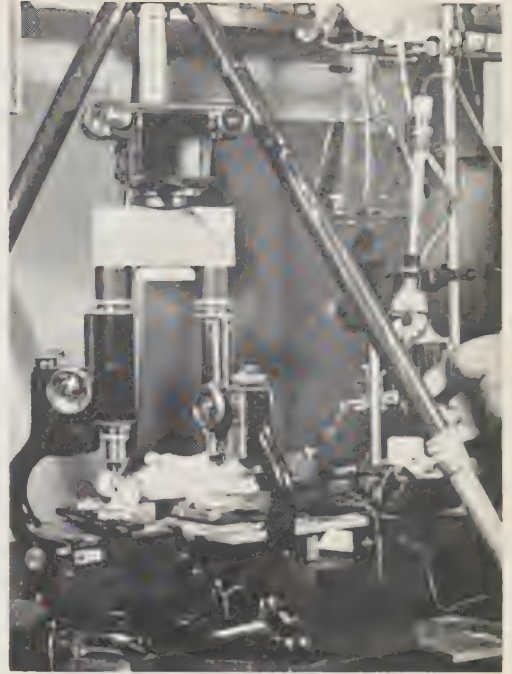


FIG. 3. Field-splitter and reflex camera set-up for photomicrography.

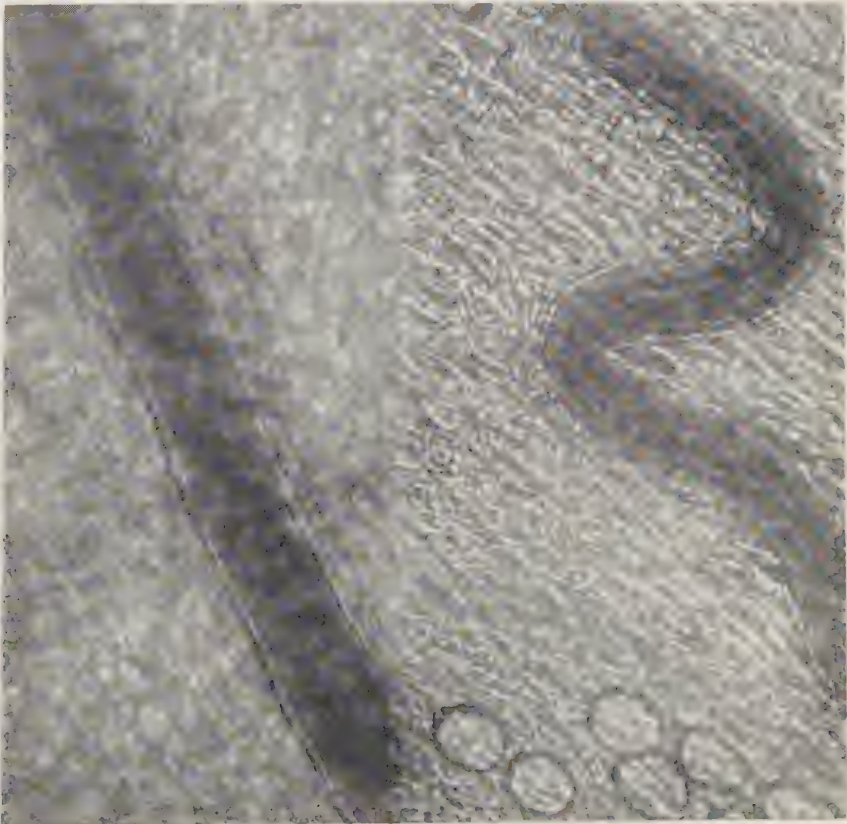


FIG. 4. Photomicrograph (147 \times). Cheek pouch vessel shown at left; mesenteric circulation at right.

placing a thermistor at the edge of the mesentery. Direct readings in degrees centigrade are indicated on a control box meter.[‡]

Still or cinephotomicrography is accomplished by placing a reflex camera above the field-splitter (Fig. 3). Images of both vascular beds are projected onto a ground glass screen from which observations are made and on which the photograph is composed before exposure. Fig. 4 is an example of the type of micrograph obtained.

For observations which need not be recorded photographically, the field-splitter is

‡ Thermistor recording unit designed and built by Mr. John Degelman, Boston Univ.

removed and a boom-mounted viewing box is centered above the microscope oculars. Two large, circular fields are thus projected side by side upon an opal glass screen. A mirror mounted above the screen at a 45 degree angle reflects the images so the observer may remain seated while viewing.

This method is currently being employed in a comparative study of vascular responses to shock and other stresses.

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Quantification of Anaphylaxis in Mice. (23634)

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It has been previously shown that a marked hematocrit rise occurs more frequently than mortality during anaphylaxis in mice(1); hence, in all probability many sub-lethal reactions were recognized. It was the purpose of the present investigation to determine if the degree of hemoconcentration in anaphylaxis is a continuous quantitative function of challenge antigen concentration.

Methods. In this study 2 different sensitizing-challenge procedures were utilized. Typical signs of anaphylaxis as previously described(1,2) were observed in both procedures. In the first procedure female Swiss-Webster mice weighing 22-27 g were sensitized with 2 IP injections (given 4 days apart) of 0.25 ml alum precipitated bovine albumin* with *Hemophilus pertussis* vaccine† (containing 1.0 mg bovine albumin, 10⁹ to 10¹⁰ pertussis vaccine cells, and 2.5% alum). Eight days after the second sensitizing injection, challenge was effected by a rapid IV injection

into the caudal vein of a 0.25 ml aliquot containing graded quantities of bovine albumin and pertussis vaccine cells. Controls included mice treated as shown in Table I. In the second procedure, mice of the same strain, sex, and weight as above were sensitized with a 0.2 ml aliquot of bovine albumin—Freund's adjuvant‡ mixture (1 part bovine albumin in saline : 1 part Freund's adjuvant), 0.1 ml of which was injected subcutaneously into each inguinal region. The total sensitizing dose contained 0.1 mg bovine albumin. Twenty-one days later, challenge was effected by injecting 0.2 ml of varying concentrations of bovine albumin in saline into the caudal vein. Controls are indicated in Table II. Hematocrits were determined as previously described(1). Blood samples were drawn from the tails of mice 5 minutes before, and 5, 10, and 15 minutes after challenge. Hematocrit change was defined from the following formula:

$$\frac{\text{Avg post-challenge} - \text{Avg pre-challenge}}{\text{Avg pre-challenge}} \times 100 = \% \text{ hematocrit change.}$$

* Bovine serum albumin, 30% sterile, for use as diluent in Rh testing. Armour Labs.

† Pertussis vaccine (Whooping Cough bacterin), Wyeth Labs., Inc. 9 protective units/ml.

‡ Bacto adjuvant, complete (Freund), Difco Labs.

TABLE I. Hematocrit Change in Mice Sensitized and Challenged by Procedure One.

| Sensitization | Challenge (mg BSA) | % hemato- crit change | % mortality (4 hr) |
|------------------|--------------------|--------------------------|-----------------------|
| APBA + HPV* | BSA + HPV* 4.0 | 46.2 ± 3.2† | 80 8/10 |
| | .38 | 47.8 ± 3.2 | 95 19/20 |
| | .125 | 38.1 ± 3.0 | 60 17/28 |
| | .050 | 36.1 ± 4.5 | 67 12/18 |
| | .030 | 33.7 ± 3.0 | 42 8/19 |
| | .025 | 33.3 ± 3.8 | 60 12/20 |
| | .020 | 26.4 ± 3.9 | 28 5/18 |
| | .015 | 14.2 ± 2.4 | 3 1/29 |
| | .008 | 24.3 ± 3.1 | 18 5/27 |
| | .004 | 11.7 ± 3.3 | 11 2/18 |
| | .002 | 6.4 ± 2.6 | 0 0/19 |
| | .001 | 1.4 ± 1.6 | 5 1/19 |
| | .0004 | -1.0 ± 3.2 | 0 0/10 |
| <i>Controls:</i> | | | |
| APBA + HPV | Saline 0 | -1.0 ± 1.0 | 5 2/43 |
| | HPV 0 | -3.0 ± 1.3 | 0 0/20 |
| APHPV | BSA + HPV 4 | -6.1 ± 1.2 | 0 0/16 |
| | HPV 0 | -1.8 ± 1.0 | 0 0/27 |
| Saline | BSA + HPV 4 | -3.9 ± 1.0 | 3 1/40 |
| | Saline 0 | -4.9 ± 1.0 | 0 0/39 |

* APBA—Alum precipitated bovine albumin. BSA—Saline solution of bovine albumin.
 APHPV—Alum precipitated pertussis vaccine. HPV—Pertussis vaccine in saline (10^9 - 10^{10}
 cells).

† Stand. error.

Results. Hematocrit changes, mortalities, and other pertinent data obtained from groups of mice treated according to the first procedure are shown in Table I. In the various control groups hematocrit change was negative in all cases and mortality at 4 hours post-challenge was insignificant. None of the 3 mice which died in the control group experi-

enced a significant hematocrit rise. It is apparent from these results that pertussis vaccine and/or the manipulations incidental to treatment did not cause either significant hematocrit rise or mortality. In the test groups it is apparent that there is a positive relationship between the degree of hematocrit change and the dose of bovine albumin. Mor-

TABLE II. Hematocrit Change in Mice Sensitized and Challenged by Procedure Two.

| Sensitization | Challenge (mg BSA) | % hemato- crit change | % mortality (4 hr) |
|------------------|--------------------|--------------------------|-----------------------|
| BSA + F* | BSA* 8.0 | 24.2 ± 3.8† | 15 3/20 |
| | 2.0 | 39.2 ± 3.4 | 22 4/18 |
| | 1.0 | 39.8 ± 3.1 | 44 4/9 |
| | .5 | 38.1 ± 2.9 | 32 6/19 |
| | .25 | 30.1 ± 3.4 | 15 3/20 |
| | .125 | 24.8 ± 3.2 | 11 2/18 |
| | .063 | 18.8 ± 2.6 | 10 3/29 |
| | .032 | 15.5 ± 4.3 | 0 0/19 |
| | .016 | 5.0 ± 2.4 | 0 0/10 |
| | .008 | 3.0 ± 1.0 | 0 0/10 |
| | .002 | -2.1 ± 1.0 | 0 0/16 |
| | .0005 | -3.9 ± 1.0 | 0 0/10 |
| <i>Controls:</i> | | | |
| Saline + F | BSA 2 | -1.8 ± 1.0 | 0 0/9 |
| BSA + F | Saline 0 | -4.0 ± 1.2 | 0 0/20 |
| Saline + F | " 0 | -2.9 ± 1.3 | 0 0/16 |

* BSA + F—Saline solution of bovine albumin emulsified in equal vol of Freund's adjuvant.
 Saline + F—Emulsion of equal volumes of saline and Freund's adjuvant. BSA—Saline solu-
 tion of bovine albumin.

† Stand. error.

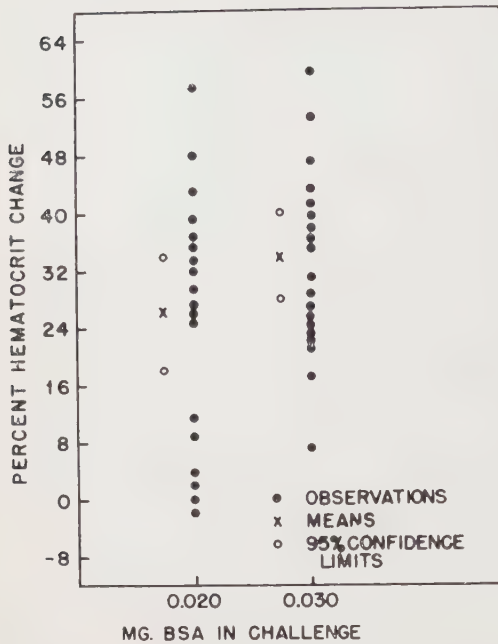


FIG. 1. Individual observations, means, and 95% confidence limits for two representative groups from Table II.

tality varies with antigen concentration in a like manner. Individual observations, means, and the 95% confidence limits, as determined by Student's T test, for 2 representative groups from Table I are plotted in Fig. 1. These observations seem to be continuous and normally distributed about the mean. Individual observations from the other test groups of Table I presented essentially the same pattern when plotted in a like manner. Hematocrit change is plotted against \log_{10}

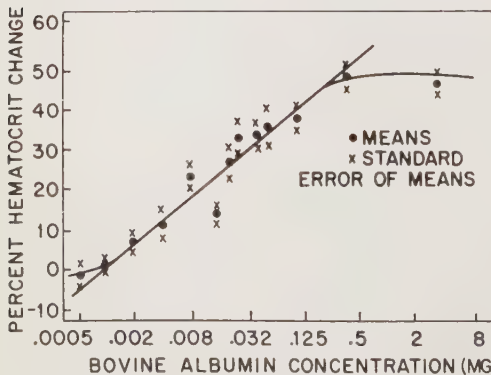


FIG. 2. Relationship of hematocrit change to \log dose bovine albumin in challenge antigen preparation. (Data from Table I.)

dose bovine albumin in Fig. 2. The relationship between the two appears to be sigmoidal. A linear regression line was fitted to the points from 0.001 mg to 0.38 mg. Above and below these points, the curve seems to level off. An analysis of variance, performed on the data between 0.001 and 0.38 mg bovine albumin, showed that the means differ significantly ($P < 0.01$) and that there is a highly significant linear trend to the relationship between \log dose bovine albumin and hematocrit change ($P < 0.001$). Significance of the deviation of individual points from the calculated regression line was determined by Student's T test. Only the mean observation for 0.015 mg bovine albumin deviated significantly ($P < 0.05$).

Hematocrit changes, mortalities, and other information in groups of mice treated according to the second procedure are shown in Table II, and hematocrit change is plotted against \log_{10} dose bovine albumin in Fig. 3.

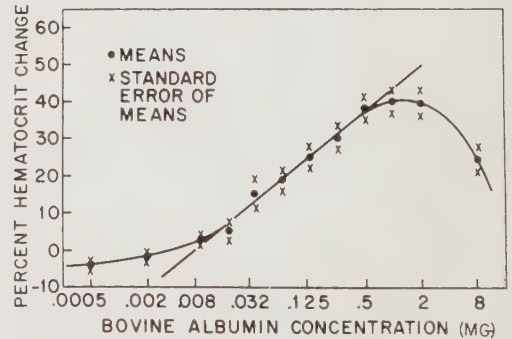


FIG. 3. Relationship of hematocrit change to \log dose bovine albumin in challenge antigen preparation. (Data from Table II.)

When individual observations were plotted (as in Fig. 1) they appeared to be normally distributed about the mean. An examination of Table II and Fig. 3 reveals that the results obtained by the second procedure are very similar to those obtained by the first procedure. Hematocrit change and mortality are positively related to challenge antigen concentration (Table II), a segment of the dose response curve could be fitted to a straight line (Fig. 3), and the range of hematocrit change and standard errors were of the same order (Tables I, II).

Discussion. Anaphylaxis in mice is cus-

tomarily measured by mortality. Data collected by this method are often inadequate because only the existence or non-existence of anaphylaxis is recognized. A method of observation which measures the extent of the reaction, rather than its mere presence or absence, is more satisfactory because variation within groups of mice can be more precisely estimated. An efficient method for determining the extent of anaphylaxis should have these characteristics: 1) There should be regularity in the dose-response relationship; 2) experimental error should be small enough so that moderate differences in the response can be measured in rather small groups of animals; and 3) individual observations should be distributed continuously so as to rule out qualitative observations. The data presented herein indicate that these criteria are approached when anaphylaxis in mice is determined by change in hematocrit. Furthermore, this method was shown to be reliable for two widely divergent anaphylactogenic procedures. This method has other advantages in that the

collection of blood samples does not seem to harm the animal; each mouse serves as its own prechallenge control, and the apparatus required is simple, inexpensive, and seems to have little intrinsic variation(1). Therefore, the use of hematocrit determinations for drawing quantitative comparisons in mouse anaphylaxis studies is recommended.

Summary. Results indicate that hematocrit rise in anaphylactic mice is a sigmoidal function of the log dose bovine albumin in the challenge antigen preparation. Over a given range, the relationship tends to be linear. Indications are that hematocrit change is a continuous function, and not a ratio of "all or none" responses. This method is recommended for drawing quantitative comparisons in studies of mouse anaphylaxis.

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Effect of Artificial Changes in Egg Composition on Hatchability and Chick Growth.* (23635)

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Egg size, as well as the weight and physical characteristics of yolk and albumen, is known to affect the hatchability of hen's eggs. The earlier work on this subject has been reviewed by Landauer(1). Lerner(2) has shown that optimal hatchability is found in eggs slightly below the mean egg size for any stock in which egg size has been favored by artificial selection.

The weight and quality of albumen in the hen's egg can be reduced artificially by local X-irradiation of the oviduct(3). The influence of these changes in the egg on hatchabil-

ity and chick growth is considered in the present report.

Materials and methods. Mature White Leghorn hens were used in these experiments. Oviducts were exposed by laparotomies and X-irradiated; other parts of the hen's body were protected from irradiation by lead shielding. The details of the irradiation procedure have been described elsewhere(3). Surgical controls were prepared for each irradiated group. In the initial experiments the entire oviducts of experimental birds were irradiated. However, this procedure resulted in such numerous and severe shell defects in eggs laid following treatment that only a few were suitable for hatchability studies. This report deals only with later experimental groups in

* This research was performed under U. S. Atomic Energy Commission contract with the University of California.

which only the magnum (albumen-producing region of the oviduct) was irradiated with 3500 r X-rays. A small amount of whole-body irradiation (<2% of the treatment dose) resulted from stray radiation from the machine and from passage of X-rays through the lead shielding. While the deleterious effect of whole-body X-irradiation is well known (4), this small quantity was considered negligible. Whole-body X-irradiation of chickens up to 300 r has been shown to have no effect on fertility or hatchability of eggs laid after treatment(5). The X-irradiation procedure used in this experiment may be considered merely as a means of producing eggs with a diminished albumen content; its results are similar to those of the more difficult method of magnum resection(6). The operative procedures used interrupted the normal sequences of ovulation and oviposition for approximately 3 weeks. When operated birds resumed lay, they were inseminated artificially at weekly intervals. The last 20 eggs of each 30 laid by each hen after treatment were incubated; the first 10 eggs were broken-out for other studies(3,7). All eggs were incubated in a forced-draft commercial type of incubator under the usual conditions of temperature and humidity. Eggs were candled twice during the incubation period; infertile eggs and those containing dead embryos were removed. Dead embryos were classified as to cause of death and age at death. Since embryos may die at such an early stage of development as to be considered infertile(8), all blastodiscs were examined carefully for any evidence of cell proliferation to distinguish between infertile eggs and early embryonic death. All chicks hatched were weighed on removal from the incubator and at weekly intervals thereafter throughout the 6-week brooding period.

Results. Response of the birds to magnum X-irradiation was variable; in some cases, a reduction of albumen quality[†] was the princi-

[†] Albumen quality is a commercial term denoting, semiquantitatively, its viscosity. Standard methods for evaluating albumen quality are available(9). Decreases in albumen viscosity increase the likelihood of contact between the shell and yolk (embryo) which may interfere with normal development.

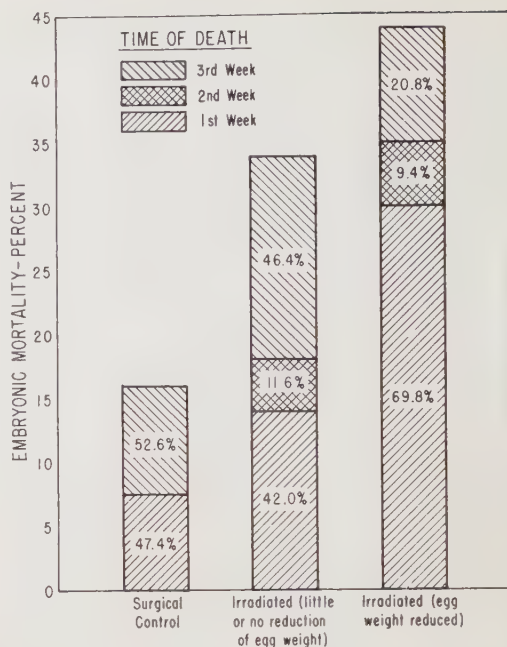


FIG. 1. Embryonic mortality in eggs laid after oviduct X-irradiation. Ordinate indicates embryo mortality observed in each of the treatment groups. Chronological distribution of embryo death (as % of total deaths) is indicated. Surviving populations of these groups are the same as in Groups 1, 2 and 3 in Table I.

pal effect, while in others marked reductions in both albumen quality and quantity were observed. In the surgical controls neither of these factors was affected adversely. The mean effect of this level of X-irradiation (3500 r) on egg composition has been reported elsewhere(3).

Accordingly, for convenience in analyzing hatchability and growth, the birds were divided into 3 groups: 1) surgical controls; 2) X-irradiated with no marked decrease in egg weight; 3) X-irradiated with egg weight decreased. Not enough experimental birds were prepared to support a more detailed analysis of X-irradiation-induced changes in egg and their effect on hatchability.

Fig. 1 indicates that total mortality, as well as its chronological distribution, was influenced by the X-irradiation procedure. There are two critical periods in chick embryo development, and under normal conditions most embryonic mortality is associated with them. The first occurs between the second and fourth days of incubation when most of the organ

TABLE I. Egg Size, Hatching Weight and Growth of Chicks.

| Treatment group | Control 1 | Irradiated; no effect on egg wt 2 | No post-treat- ment reduction of egg size 1 & 2 | Irradiated with reduction of egg size 3 |
|--|-----------------|--|--|--|
| No. hens | 7 | 7 | 14 | 11 |
| No. chicks hatched | 86 | 84 | 170 | 66 |
| Egg wt (g)—Pre-treatment | 59.1 \pm 2.0 | 58.3 \pm 1.8 | 58.7 \pm 1.3 | 58.0 \pm .8 |
| Post-treatment | 61.2 \pm 1.6 | 59.8 \pm 1.7 | 60.5 \pm 1.1 | 53.5 \pm 1.0 |
| Chick wt—g | 41.9 \pm .4 | 40.6 \pm .5 | 41.2 \pm .3 | 35.6 \pm .4 |
| % egg | 68.0 \pm .4 | 66.0 \pm .4 | 67.0 \pm .3 | 65.5 \pm .5 |
| Body wt of chick as multiple of hatching wt at: | | | | |
| 1 wk | 1.67 \pm .03 | 1.64 \pm .02 | 1.66 \pm .02 | 1.74 \pm .02 |
| 2 | 2.96 \pm .06 | 2.67 \pm .10 | 2.80 \pm .06 | 3.19 \pm .06 |
| 3 | 4.75 \pm .11 | 4.71 \pm .09 | 4.73 \pm .07 | 5.32 \pm .10 |
| 4 | 6.97 \pm .15 | 7.10 \pm .20 | 7.04 \pm .12 | 7.96 \pm .22 |
| 5 | 9.90 \pm .21 | 9.86 \pm .24 | 9.88 \pm .16 | 10.86 \pm .25 |
| 6 | 13.05 \pm .43 | 13.50 \pm .36 | 13.39 \pm .29 | 14.90 \pm .41 |

systems are formed. Any developmental deficiency results in the death of the embryo, and ordinarily this accounts for slightly more than one-third of the total embryo mortality. The second critical period occurs between the 17th and 21st days of incubation (*i.e.*, the last four days of that process) when various pre-hatching changes take place. Ordinarily, about two-thirds of total embryo mortality occurs during this period.

Eggs in which albumen quantity and quality were reduced were characterized by a marked increase in mortality during the first week of incubation. An examination of embryos dying in this period indicated that much of this mortality resulted from a failure in very early differentiation, rather than the more commonly observed later defects in organogenesis. The occurrence of appreciable second-week mortality was also unusual. Mortality associated with the terminal period of development, however, was of the type normally expected (*viz.*, failure of the embryo to orient properly, late yolk sac absorption, difficulties in change from allantoic to pulmonary respiration, etc.).

Differences in hatch weight (absolute and relative to egg weight) were found between the chicks from control and irradiated hens (Table I). These differences are large only between the controls and the group in which egg weight was decreased. Although the ob-

served differences between the groups in relative chick weights (calculated as percent of egg weights) are statistically significant, they are small. Other investigators, whose results have been reviewed by Hutt(10), have consistently found that within a single breed day-old chick weight is determined largely by egg weight.

Whether or not differences in the growth rates of the 3 groups of chicks exist was found to depend upon the basis of the comparison (*i.e.*, absolute or relative to hatch weight). There is no systematic difference by either comparison between the control chicks (Group 1) and those from X-irradiated hens in which egg size was not markedly decreased (Group 2). The growth rate (relative to hatch weight) was significantly greater in the chicks from the X-irradiated hens in which egg size was reduced (Group 3). However, on an absolute basis, there is no statistically significant difference in the growth rate after the first week of brooding.

Discussion. Decreases in albumen quality and quantity brought about by oviduct irradiation are associated with a decreased hatchability. This artificial alteration, however, does not necessarily relate to the effects of natural variations in albumen size and quality on hatchability. The effect of local oviduct X-irradiation on the egg, and especially the adverse effect on albumen quality, is simi-

lar to that of certain respiratory diseases(3). Hatchability of eggs from flocks after an outbreak of respiratory disease, such as Newcastle disease, is decreased, and characteristically very early embryonic mortality, similar to that reported here, is observed.

Other conditions leading to higher embryo mortality do not follow the pattern observed after qualitative or quantitative decreases in the albumen (*i.e.*, oviduct X-irradiation or respiratory disease). The embryo mortality encountered after excessive inbreeding, for example, is much more variable, but deaths occur mainly at the usual critical periods of development.[‡] No reproducible pattern is associated with this cause of embryo death.

Increased embryo mortality in the second week of incubation is also encountered in eggs from birds deficient in certain specific nutrients (*viz.*, riboflavin(11)) and in some stocks which carry specific genetic lethals. These intermediate deaths may result from metabolic abnormalities.

It appears that the "growth potential" of the chicks is not affected by the X-irradiation treatment of the dams. Although chicks from the lighter eggs are smaller at hatching, this difference soon disappears. Similar results have been obtained with rats which were stunted early in life by a protein-deficient ration(12). Upon return to a normal feeding regime, stunted rats made up their early deficiency and eventually out-grew their controls. Several studies with normal birds(10)

have also indicated that the size of the chick at hatching has little effect on its subsequent growth.

Summary and conclusions. Artificial decrease in albumen quality and quantity caused by local X-irradiation of the oviduct reduces the hatchability of eggs. The distribution of embryo mortality is different from that encountered in normal eggs: very early mortality is markedly increased. Chicks from such eggs have a lower hatch weight; however, no effect on subsequent chick growth was observed.

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Lack of Correlation Between Skin Test and "Tissue" Sensitivities in Guinea Pigs Infected with *Brucella abortus*. (23636)

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The problem of a negative or a depressed skin test for bacterial hypersensitivity of the delayed type in animals known to be infected with the test agent is a very real one. Many reports have dealt with changes in skin sensitivity to tuberculin in humans and other animals infected or presumably infected with *Mycobacterium tuberculosis* during intercurrent infection(1,2), during pregnancy(3,4), during administration of drugs(5), and during changes in age of animals(6) to mention only a few. Data which are derived both from *in vivo* skin tests and *in vitro* "tissue sensitivity tests" are rather scarce(5,7).

During studies on host-parasite relationships using *Brucella abortus* and guinea pig monocytes(8), we found that many guinea pigs which had received large, infecting doses of living *Brucella abortus* did not exhibit positive skin tests after a suitable period of time despite the fact that at autopsy these same animals almost invariably showed splenic and other lesions due to *B. abortus*. We decided to study this phenomenon using *in vitro* techniques for determination of "tissue" sensitivity to ascertain if there was a discrepancy between the tuberculin type skin test and *in vitro* "tissue" sensitivity. In recent studies(9), Heilman, Howard and Carpenter reported an *in vitro* system for the study of "tissue" sensitivity in brucella infected animals. Our findings confirm and extend those of these authors.

Materials and methods. Guinea pigs were young adults at the time of infection with *B. abortus*. Strain Lo or strain SA-S‡ was injected subcutaneously in a dose of 0.1 ml of

a saline suspension (BaSO₄ standard #1) of a 48 hour culture. Animals were killed at stated times and the spleens were removed immediately. Tissue culture methods were those described in a previous paper(10). In some cases pooled, fresh, normal guinea pig serum (30%) and Hanks' balanced salt solution (70%) were used as the medium while in other cases fresh autologous serum and Hanks' comprised the medium. No differences due to this factor were noted. Splenic fragments were imbedded in a chicken plasma clot by using a drop of chick embryo extract as the clotting agent. The proper amount of bacterial extract in 0.5 ml of medium was added as soon as the clot had become firm. Eight tubes, each containing 2 explants, were used for each treatment. The extract of brucella has been described by Pomales-Lebrón (12). For a portion of the experiments an extract of strain Lo was used. An extract of SA-S was used for others. Results were similar with either extract. Saline in place of extract was added to control culture. Cultures were incubated at 37° for 4 days. Skin tests were carried out by intradermal injection of 0.1 ml of a 1:10 dilution of the brucella extract. Certain animals giving negative skin test at this dilution were retested using a higher concentration of extract. After preparation of the explants, some of the spleens were cultured on tryptose agar to determine if viable brucella were present. Crude estimates of the number of recoverable organisms were made (Table I). The statistical significance of differences between extract-containing tissue cultures and saline-containing cultures, based on the migration and degeneration of large wandering cells, was determined according to procedures reviewed by Boyd (11). A *p* value of 0.05 or less than 0.05 indicates the cultures to which extract had been added showed significantly less migration and

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TABLE I. Results of *In Vitro* "Tissue" Sensitivity Tests and Bacterial Cultures of Normal and Infected Guinea Pigs.

| Animal | <i>B. abortus</i> strain | Duration infection | Skin test | Tryptose culture of spleen | Probability that control and exp. showed no difference | | |
|-------------|--------------------------|--------------------|-----------|----------------------------|--|--------------|--------------|
| | | | | | Control vs | | |
| | | | | | 1:25 extract | 1:50 extract | 1:75 extract |
| Normal | | | Negative | N.D. | | p > .05 | |
| " | | | " | " | | p > .05 | |
| Infected | Lo | 40 days | Positive | " | | p < .01 | |
| " | " | 3 mo | " | " | | p < .01 | |
| " | " | 5 " | Negative* | " | | p < .05 | |
| Normal # 33 | | | " | Negative | p > .05 | | p > .05 |
| Infected 45 | SA-S | 11 days | " | ++++ | p > .05† | | p > .05 |
| " 30 | " | 1 mo | " | ++ | p = .01 | | p > .05 |
| " 337 | " | 5 " | " | + | p = .01 | | p = .01 |
| " 338 | " | 5 " | Doubtful | Negative | p = .05 | | p = .05 |
| " 494 | " | 5 " | Negative | + | p > .05 | | p > .05 |

In one instance tissue from a normal noninfected animal was inhibited at 1:75 conc. of extract. No reason can be given for this discrepancy. The high probability ($p = .01$) left no doubt as to the effect. This had never been observed before during titration of the allergen.

N.D. = not done. ++++ to + refer to relative amount of growth of *B. abortus* from spleen cultures.

* Tested 2 mo and 4 mo. Negative both times. Cells were, however, capable of suppressing growth of *B. abortus* as would be expected of immune cells (Pomales-Lebrón and Stinebring(8)).

† Probably before allergy had developed.

significantly more degeneration than the controls.

Results. From the data in Table I one may conclude: (a) a definite cytotoxic effect of brucella extract on splenic cells from infected guinea pigs has been observed, (b) a negative skin test is no criterion of "tissue" sensitivity in infected animals, and (c) skin sensitivity and "tissue" sensitivity may not be exhibited even though brucella may be isolated from the host 5 months after infection.

The first conclusion is similar to that of Heilman *et al.*(9) in regard to brucella infections of guinea pigs. The reaction is probably due to the same mechanisms as in the experiments first described by Rich and Lewis(13).

The work of Lurie *et al.*(5) and Heilman and Feldman(7) clearly demonstrates that "tissue" sensitivity and skin sensitivity in the case of experimental tuberculosis are not necessarily correlated. However, the animals studied were treated with hormones or were in the final stages of progressive disease.

Our findings indicate, similarly, that the two tests may be dissociated in experimental brucellosis in untreated animals exhibiting no evidence of intercurrent infections and apparently tolerating the experimental infection well (guinea pigs do not ordinarily succumb

to *B. abortus* infection). Therefore, the *in vitro* test is a better indication, in most cases, than is the skin test of sensitivity of the host to a particular allergen. However, it must be noted that in one guinea pig in our series no "tissue" sensitivity could be found and the skin test was negative, but organisms from an infection of 5 months duration could be isolated from the spleen. This requires further confirmation.

Summary. Evidence is presented to show that guinea pigs experimentally infected with *Brucella abortus* and which do not show a positive skin test upon intradermal injection of bacterial extract usually possess a "tissue" sensitivity to this antigen.

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Propagation of Measles Virus in Cultures of Chick Embryo Cells.* (23637)

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Cultivation of measles virus in cultures of chick embryonic tissues and in developing hens' eggs has been reported by a number of workers. Others in similar attempts failed to obtain convincing evidence that the agent could be thus propagated(1,2,3,4,5). These discrepancies depended at least in part upon the fact that in nearly all these studies the only criterion of viral multiplication formerly available in the laboratory consisted in the development of signs of measles in monkeys following inoculation of materials suspected to contain the virus. Since a varying proportion of "normal" monkeys does not develop overt disease even when inoculated with materials known to contain the agent(1,6), it is understandable that the findings of different investigators were not always in agreement.§ In 1954, a more dependable index of viral

multiplication was recognized by Enders and Peebles in the characteristic changes which the virus produces in primate epithelial cells (7). Accordingly, a reinvestigation of its capacity to multiply in chick embryonic tissues was undertaken.

At first attempts were made to cultivate in embryonated eggs several strains of measles virus that had been subjected to only a few passages in human kidney cells. No evidence was obtained that any of them multiplied in this host. One of these strains (Edmonston) was added to cultures of chick embryo tissues prepared according to the procedure that Plotz described(8), and with which Rake and his co-workers also reported successful results (3). But again no indication of increase or even persistence of the virus was noted(7). Ultimately adaptation to chick embryos of this strain was attained by Milovanovic, Enders and Mitus(9) after many passages in human renal and amnion cells. In preliminary experiments this chick embryo-adapted virus was found to multiply readily in cultures of chick cells(9), where its behavior will now be described in detail.

Materials and methods. Virus. The Edmonston strain was isolated from the blood of a child in the early acute phase of measles (7). When this investigation was initiated it had been passed serially 24 times in human kidney and 28 times in human amnion cell cultures, and 6 times in embryonated eggs.

Storage of virus. Virus suspensions in the form of infected tissue culture fluids were

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§ At least one of the factors responsible for variation in response of monkeys has lately been defined. Thus Peebles and his co-workers(6) have demonstrated in a high proportion of "normal" rhesus and cynomolgus monkeys maintained under laboratory conditions neutralizing and complement fixing antibodies that react specifically with measles virus. These antibodies presumably arise during the course of unobserved spontaneous infections with this virus or a closely related agent.

stored in the CO₂ cabinet at approximately -58°C. *Titration of infectivity.* Tenfold dilutions of infected tissue culture fluids were made in bovine amniotic fluid medium (BAF see below). Aliquots of 0.1 ml of each dilution were added to 3 tissue cultures. The cultures were examined at frequent intervals for cytopathic changes. Final readings were made as routine on the 14th day after addition of the inoculum. Titers are expressed as log TCD₅₀/0.1 ml as calculated by the method of Reed and Muench. *Neutralization tests.* Acute and convalescent phase sera from a patient with measles and sera taken from a cynomolgus monkey before and 25 days after inoculation with the Edmonston strain of the 23rd passage in human kidney cell cultures (6), were employed. The sera were inactivated at 56°C for 1/2 hour and a series of dilutions increasing by a factor of 2 were prepared in isotonic phosphate buffer solution. To each dilution an equal volume of virus suspension containing a calculated 200 TCD₅₀/0.1 ml was added. The mixtures were allowed to stand for 1 hour at about 6°C. 0.1 ml of each was then added to each of 3 cultures. Serum neutralization titers were calculated by the method of Reed and Muench from readings of cytopathic changes taken on the 14th day. *Tissue cultures.* Cultures of trypsinized human amnion and human kidney cells were prepared and maintained according to procedures previously described(9). Roller tube cultures of fragments of whole 9-day chick embryos were initially used and later cultures of trypsinized chick embryo cells. The latter were prepared according to the technic of Dulbecco(10) as modified by Dr. Donald N. Medearis, Jr. in this laboratory. Embryos from eggs incubated at 100°F for 7 to 9 days were washed in phosphate buffer solution after removal of the eyes and transferred to an Erlenmeyer flask containing about 50 ml of 0.25% trypsin solution at pH 7.8. The flask, after standing for 30 minutes at 37°C or for 60 minutes at room temperature, was shaken for 1 minute. The suspension of cells was separated from the remaining coarse debris and centrifuged at 1000 rpm for 10 minutes. The fluid was discarded and replaced with an equal

volume of BAF medium. After deposition in the centrifuge the cells were resuspended in this medium to yield a concentration of 200,000 to 400,000 per ml. Sufficient cells were usually obtained from a single embryo to provide about 100 cultures. Aliquots of 1 ml of the cell suspension were added to 150 x 16 mm test tubes, which were set at an angle of about 5° and incubated for 48 to 72 hours at 37°C when suitable monolayers were formed. The medium was changed before addition of virus and at intervals of 7 to 10 days thereafter. *Tissue culture medium.* Bovine amniotic fluid medium containing 5% normal horse serum was employed as routine(9) for growth and maintenance of cultures of chick cells. During the phase of outgrowth human amnion cells were nourished with a horse serum-glutamine salt mixture(9) and thereafter with BAF medium.

Experimental. Multiplication in cultures of chick embryonic cells. A series of passages in chick cell cultures was initiated with a 10% suspension of amniotic membranes from the 6th chick embryo passage of the Edmonston strain(9). Infectivity titer of this suspension in human amnion cells was 520 TCD₅₀/0.1 ml. Three roller tube cultures of chick tissues were each inoculated with 0.1 ml of the suspension. A pool of undiluted fluids removed on the 19th day was used as inoculum for the 2nd passage in roller tube cultures. The 3rd passage was initiated in trypsinized chick cell cultures with a pool of fluids collected on the 14th day from cultures of the 2nd passage. Thereafter trypsinized cell cultures were inoculated with pooled fluids from the previous passage taken 7 to 14 days after virus was added. Infectivity of fluids removed successively during each passage, with the exception of the second, was assayed in human amnion cells. In Fig. 1 the infectivity titers of fluids from the 5th and the 8th passages are presented graphically to show how the virus increased and declined. The curves differ significantly in respect to the time at which the maxima were attained. Maximal titers determined during each of 19 serial passages are listed in Table I with the times at which the fluid was harvested. In 11 of the first 12 passages the titers represent the true

maxima within the limits of the experimental conditions. From the later passages only fluids taken during the first 11 to 14 days were tested. In these instances the titers may not represent the true maxima, although this seems probable, since they are within the range of the highest observed in this or in any other system we have employed (7,9).

The data included in Fig. 1 and Table I show that the egg-adapted measles virus multiplied readily in this system. That the biological properties of the agent were in certain respects modified to meet the conditions presented by this new environment is suggested by inspection of the various times at which it was present in highest concentration. Thus during the first 6 passages for which information is available the viral content of the fluid did not reach its peak until about the 30th day following inoculation. (Average: 27 days.) In contrast the maxima for the next 6 passages, with one exception, were reached within 14 days or less (Average: 12 days). Additional evidence of an altered biological characteristic emerging during these passages is to be found in the assumption of cytopathogenicity for chick cells.

Cytopathogenic effects in chick cells. Throughout the first 4 serial passages no changes definitely attributable to the virus were noted. Its presence and increase were determined solely by effects produced in human amnion cells exposed to fluids taken from

TABLE I. Maximal Titers Found in Fluids of Serial Passages of Measles Virus in Chick Embryo Tissue Cultures.

| Passage | Titer* ($\text{TC}_{50}/0.1 \text{ ml}$) | Days after inoculation |
|---------|---|---------------------------|
| 1 | 2.5 | 27 |
| 2 | VP† | |
| 3 | 3.5 | 33 |
| 4 | 3.8 | 28 |
| 5 | 4.5 | 30 |
| 6 | 4.5 | 29 |
| 7 | 4.8 | 13 |
| 8 | 5.3 | 9 |
| 9 | 3.8 | 7 |
| 10 | 3.8 | 11 |
| 11 | 3.5 | 19 |
| 12 | 4.8 | 14 |
| 13 | 4.8 | 6 |
| 14 | 4.5 | 11 |
| 15 | 4.3 | 9 |
| 16 | 4.8 | 11 |
| 17 | 3.8 | 8 |
| 18 | 4.3 | 10 |
| 19 | 4.8 | 7 |
| 20 | 4.3 | 7 |

* As determined in cultures of human amnion cells.

† Presence of virus confirmed but concentration not determined.

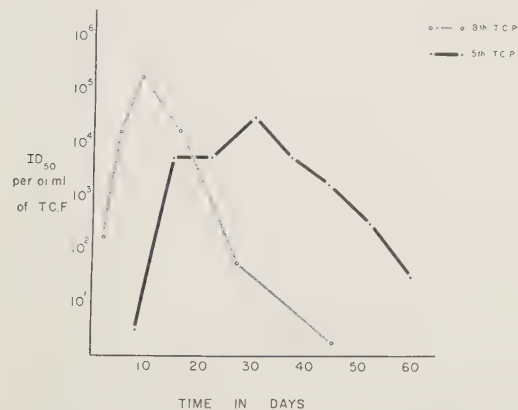


FIG. 1. Infectivity titers as determined in cultures of human amnion cells of fluids removed at various intervals from the 5th and 8th passages of measles virus in chick embryo cell tissue cultures.

chick cell cultures. On the 8th day of the 5th passage distinctive changes became evident, consisting of increased refractility of cells in circumscribed areas. Many affected cells exhibited a fusiform or stellate configuration owing to the formation of filamentous cytoplasmic processes, which varied in length, and often appeared to be finely beaded. In Fig. 2 A is shown such changes in cells of the 22nd passage 10 days after addition of virus. The appearance of cells in a control culture is illustrated in Fig. 2 B. In fixed and stained preparations eosinophilic intranuclear and cytoplasmic inclusion bodies were observed that closely resembled those found in infected primate cells (7,9,11) as shown in Fig. 3. As the process advanced the affected cells became rounded, pyknotic and eventually disintegrated. In succeeding passages these changes appeared sooner and proceeded more rapidly to involve a large proportion of the cell population. Complete destruction of all cells, however, even after many weeks has not been observed and virus production has continued at low levels. It is noteworthy that shortly after the cytopathogenic effect became evi-

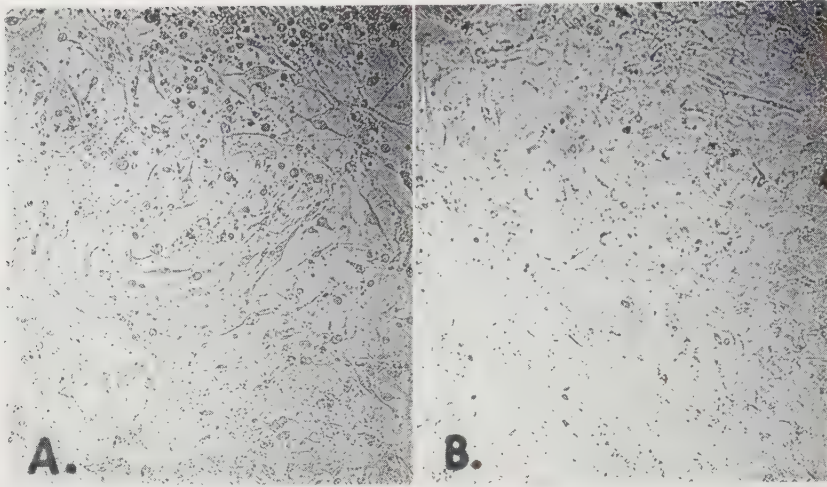


FIG. 2A. Cytopathic changes in cells 10 days after addition of measles virus. Edmonston strain 22nd passage in chick cell tissue cultures. Unstained. Mag 147 \times .

FIG. 2B. Cells in control culture maintained under same conditions but without addition of virus. Unstained. Mag 147 \times .

dent the time required by the virus to attain its maximal concentration in the fluid was reduced.

Identification of the virus. The agent propagated under these conditions was identified as measles virus by its behavior in cultures of human amnion cells and in neutralization tests with acute and convalescent phase measles sera. Addition of infected fluids from chick cell cultures of all passages to primary cultures of human amnion cells was followed by the appearance of cytopathic changes characteristic of measles virus(9). Infectivity titers of the fluids from later passages as determined simultaneously in amnion and in chick cells, were of the same magnitude as shown in Table II. These findings indicate that the same agent was responsible for the

TABLE II. Infectivity Titers of Fluids from Various Passages of Virus in Chick Cells as Determined in Cultures of Chick and Human Amnion Cells.

| Passage | Day fluid harvested* | Titer† | |
|---------|----------------------|--------|--------|
| | | Chick | Amnion |
| 6 | 14 | 3.8 | 3.8 |
| 7 | 13 | 4.5 | 4.8 |
| 8 | 9 | 5.5 | 5.3 |
| 10 | 11 | 4.5 | 3.8 |
| 13 | 6 | 4.3 | 4.8 |
| 17 | 8 | 4.3 | 3.8 |

* Day after inoculation fluid harvested from serial passages of virus in chick cell cultures.

† TCD₅₀/0.1 ml.

TABLE III. Results of Neutralization Tests with Human and Monkey Measles Sera and Virus of the 7th Passage in Chick Cells.

| Cells used in test | Serum | Serum titers* | |
|--------------------|--------|-----------------------|--------------|
| | | Acute or preinfection | Convalescent |
| Human amnion | Human | 7 | 710 |
| Chick | " | <8 | 840 |
| Human amnion | Monkey | <4 | 575 |

* Reciprocal of final dilution of serum inhibiting cytopathogenic effect of the virus in one-half of the cultures as calculated by the method of Reed and Muench.

cytopathogenic effects observed in both kinds of cells.

The results of neutralization tests are summarized in Table III. The acute phase serum from a measles patient possessed little capacity to inhibit the cytopathogenic effects of the agent in either human amnion or chick embryo cells; the convalescent phase serum prevented these effects in high dilution. Similar results were obtained in amnion cell cultures with sera of a monkey taken before and after inoculation of the Edmonston strain before adaptation to the chick embryo.

Antibodies in rabbits with virus propagated in chick cells. It was determined that antibodies reacting specifically with the virus were induced in rabbits by injection of fluid from infected cell cultures. Two animals of 6 and 7 pounds respectively were inoculated with

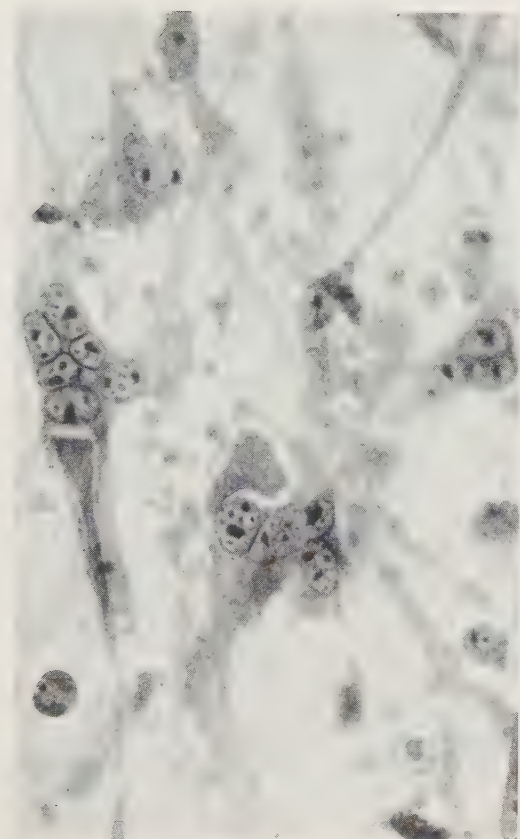


FIG. 3. "Multinuclear giant cells" in a culture of chick embryo cells 19 days after addition of measles virus, Edmonston strain, 7th passage in chick cell tissue cultures. Bouin's fixative; collodion imbedding; H & E stain. Mag 880 \times . Note eosinophilic intranuclear inclusions and large irregular cytoplasmic masses.

undiluted fluid removed on the 8th day from a 15th chick-cell passage. To minimize the development of antibodies against other constituents, the following medium was employed instead of the usual BAF mixture:

| | |
|--|---------------|
| Hanks' balanced salt solution (with phenol red) | 50% |
| Chick amniotic fluid (from 8 day eggs) | 50% |
| Penicillin | 50 units/ml |
| Streptomycin | 50 μ g/ml |
| Mycostatin | 50 " |

The procedure recommended by Ramos-Alvarez and Sabin for production of antisera to ECHO viruses(12) was followed. Neutralization tests were carried out in cultures of human amnion cells with the rabbits' sera

drawn just before immunization and 24 days thereafter. The Edmonston virus used in these tests had been passed 26 times in human renal cells and then 29 times in human amnion cells but had not been cultivated in chick cells. Neutralizing antibodies were not detected in the initial specimen of serum of either rabbit in a final dilution of 1:4. The titers found for the 24th day specimens were respectively 1:128 and 1:180. These results indicate that anti-measles antibodies can be produced in rabbits by inoculation of the chick cell-adapted virus. They also afford additional evidence for the identity of the chick-cell-adapted virus and the agent originally isolated from a patient with measles.

Cultivation with media of known composition. Chick cells originally grown in BAF medium were washed 3 times with Hanks' solution at pH 7.6 and divided into 4 sets each of 6 cultures. Each set was maintained with one of the following solutions:

- (a) Hanks' balanced salt sol
- (b) Hanks' sol, 3 parts; ox-serum ultrafiltrate, ||
1 part
- (c) Medium 199 (Morgan and Parker)
- (d) BAF medium

To each of 3 cultures in each set was added 0.1 ml fluid from the 20th chick-cell-passage of the virus. (Titer = $10^{4.3}$ TCD₅₀/0.1 ml in human amnion cells.) Cytopathic changes were apparent on the 4th day in all tubes to which virus was added. The fluids from infected cultures in each set were collected and pooled on the 7th day. At this time the cells in the control cultures remained in fair to good condition. The titer of each pool in human amnion cell cultures was determined as follows:

| Medium | TCD ₅₀ /0.1 ml |
|--------|---------------------------|
| (a) | $10^{2.5}$ |
| (b) | $10^{2.8}$ |
| (c) | $10^{3.3}$ |
| (d) | $10^{4.3}$ |

While the quantity of virus in cultures with medium 199 is smaller, it nevertheless approaches that often encountered in those of primate or chick cells maintained with BAF medium. It is unlikely that in this experi-

ment the virus found in the fluids represents that introduced as inoculum. In unpublished experiments we have observed in agreement with data reported by Ruckle(13) that decay of measles virus at 37°C is very rapid. Therefore if the agent had failed to multiply one would not expect to encounter it in appreciable quantities at the end of 7 days.

Complement fixing antigen in cultures of chick cells. Antigen fixing complement with antibody developing during convalescence from measles regularly appears in the fluid of infected primate cell cultures(7,9). Repeated attempts have so far failed to demonstrate this antigen in the fluid of infected chick cell cultures. Recently, however, in a preliminary experiment antigen reacting specifically with convalescent measles serum has been prepared by repeated (3 times) freezing and thawing of whole cultures infected 40 days previously with the virus. It is probable, therefore, that complement fixing antigen present in the cells is released by this procedure.

Discussion. It is not surprising that measles virus previously adapted to the chick embryo proliferates in the cells of this host when they are maintained in culture. For most if not all viruses capable of multiplying in the developing egg can also be propagated *in vitro* in cells derived from its tissues. Accordingly, the principal significance of these results lies elsewhere. First, they confirm, at least in principle, the early findings of Plotz and Shaffer and Rake. The only indicator then available in the laboratory of the presence of this agent was the development of overt disease in the monkey. The data here presented were obtained through the application of criteria for its recognition which appear to be more reliable and specific. There is, therefore, little reason longer to doubt the conclusion of these workers that the measles virus can be cultivated in chick embryo cells either *in vivo* or *in vitro*.

We have not, however, succeeded in reproducing their results under conditions approximating those which they employed. Thus their original inocula consisted of virus present in materials derived from patients or mon-

keys with measles and it would seem *a priori* that in this state the agent would tend to behave more like our strains which after isolation were subjected to only a few passages in primate cells. As previously emphasized, we have repeatedly failed to maintain such strains in either chick embryos or chick cell cultures. Perhaps this remaining discrepancy will be resolved when the effect of inoculating chick cells with blood or throat washings of measles patients is determined by means of the newer criteria for viral activity. For possibly a few passages of the virus in primate cells may interfere with its adaptation to chick embryo systems. If so, any effect of this sort must be transitory, since we have succeeded in cultivating the virus in the chick embryo only after it has undergone many passages in human epithelial cells.

The acquisition of cytopathogenicity for chick cells in culture is also of interest. Its sudden emergence during the 5th serial passage at first suggests a mutation. It may, however, reflect the capacity of the agent to increase more rapidly within the cells of this unnatural host which was gained at about the same time. Thus the phenomenon, if it can be elicited with regularity, might prove useful in the future analysis of the factors that in general underlie viral cytopathogenicity.

From the practical standpoint the development of cytopathogenicity is of value since it makes possible the application of a technic to the assay of infectivity and the titration of antibodies which is more convenient, rapid and economical than the culture of primate cells. A greater utilitarian significance, perhaps, may be the demonstration that quantities of virus are produced in chick cells even when maintained with a medium of known composition that is comparable to the concentrations found in cultures of primate cells. Fluids from such cultures might provide appropriate material for the preparation of vaccine. Such vaccine would contain no foreign proteins or other macromolecular substances save the small amounts derived from the chick cells. That chick embryo proteins in low concentration may be injected with very little risk is indicated by extensive experience in

man with yellow fever or influenza virus vaccine prepared in eggs.

Summary. An egg-adapted strain of measles virus has been propagated in cultures of chick embryo cells throughout 24 serial passages. Beginning with the 6th passage an abrupt decrease occurred in the time required to attain maximal concentration of virus in the fluid. The concentration of virus was comparable to that found in cultures of infected primate cells. In the 5th chick cell passage the virus began to produce cytopathic changes closely resembling those occurring in human epithelial cells infected with measles virus. The identity of the agent was confirmed in neutralization tests with measles antisera. In chick cells maintained with a medium of known composition virus production approached that in cultures nourished with serum and tissue extracts. Inoculation of rabbits with the virus propagated in chick cells was followed by development of neutralizing antibodies specific for the agent of measles.

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Increase of Urinary Citrovorum Factor Activity in Patients Receiving Methotrexate (Amethopterin) (23638)

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Regression of metastatic trophoblastic tumors has been observed in women treated with methotrexate, a folic acid antagonist(1). The mechanism of action of the folic acid antagonist has been shown to include an inhibition of the synthesis of citrovorum factor (CF) in liver and prevention of its utilization by tissues in laboratory animals(2,3). It, therefore, was of interest to attempt to correlate the daily excretion of urinary CF activity with tumor response in patients with trophoblastic tumors receiving methotrexate therapy.

Methods. All patients received a constant diet composed of a liquid mixture of milk, cream, eggs and dextrose during the period of study. The mixture contained approximately 1,000 calories, 5 μ g of folic acid and 6 μ g of

CF/liter. The dietary intake of these patients ranged from 1,800 to 2,500 calories/day. Daily 24 hour urines were carefully collected and refrigerated. 25-35 mg of methotrexate was given daily for 5 days either by mouth or intramuscularly. Toxicity, manifested by anorexia, nausea, oral ulcerations and occasional emesis was present but did not interfere with the constant dietary intake. The CF activity was assayed by the microbiological technic of Sauberlich and Baumann (7) using Difco assay medium. Turbidimetric readings were made after 16 to 18 hours incubation at 37°C. Since *Leuconostoc citrovorum* #8081 (*Pediococcus cerevisiae* ATCC 8081) was susceptible to methotrexate inhibition, a methotrexate resistant strain *L. citro-*

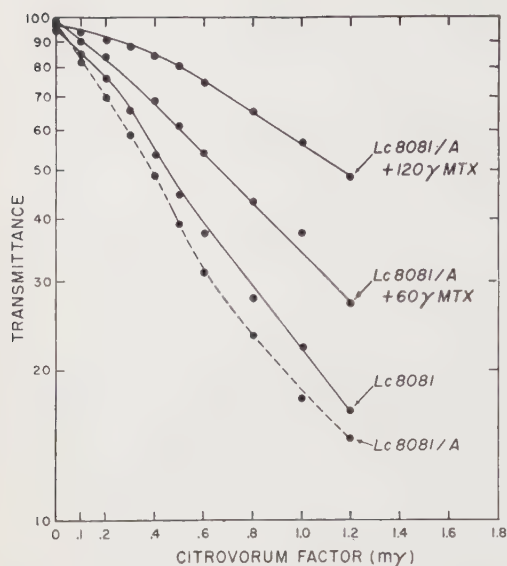


FIG. 1. Growth curves of *Leuconostoc citrovorum* #8081/A.

vorum #8081/A obtained from Hutchinson's laboratory(4) was used as the test organism. All assay tubes had a total of 6 ml containing 15 μ g of methotrexate/tube. *Leucovorin* (synthetic DL 5 CHOTHFA) was used as a reference standard. The amount of CF referred to throughout these studies was calculated on the basis of naturally-occurring material this being twice as active as leucovorin(8). In order to obtain maximal CF activity, 2 parts of urine were mixed with 1 part of 5% sodium ascorbate solution at pH 6.0 and autoclaved for 10 minutes at 120°C before assay(5). Whatman #1 paper strips of 1 inch width and 15 inches in length, 0.1 M phosphate buffer at pH 7.6 and descending technic were used for chromatographic separation of CF. The R_f value for CF activity was usually between .70-.75 with this solvent system. The urinary nitrogen was determined by the standard micro-Kjeldahl method.

Results. The growth curves of *L. citrovorum* #8081/A with and without methotrexate are shown in Fig. 1. It is noted that this variant maintains its dependence on CF logarithmically for growth stimulation. Methotrexate alone, 60 μ g, and 120 μ g/tube did not support growth of *L. citrovorum* #8081/A in the absence of CF. Instead, there was some inhibition of growth

even in the presence of CF as was originally observed by Hutchinson. However, it is an appropriate organism for assaying CF in the presence of methotrexate provided the methotrexate concentration does not go beyond 40 μ g per assay tube. The metabolic studies on patient R.S., a 34-year-old man with metastatic embryonal carcinoma and choriocarcinoma to lungs and abdomen is illustrated in Fig. 2. It is noted that the urinary CF activity was fairly constant on the days when the patient received no methotrexate. When methotrexate was given, urinary CF activity rose to 3-5 times that of the control values. These findings were confirmed by chromatographic separation and assaying with *L. citrovorum* #8081. The rise of CF activity occurred within the first 24-hour period and subsided in similar fashion after the drug was discontinued. Change of urinary nitrogen excretion was not marked, although there was a suggestion of an increase of nitrogen excretion from 0.5 to 1 g on the days when the patient received methotrexate. In spite of the marked increase of urinary CF activity there was no significant favorable effect on this patient's disease.

The studies on patient A. A., a 28-year-old woman with extensive choriocarcinoma of uterus and with metastases to the lungs is il-

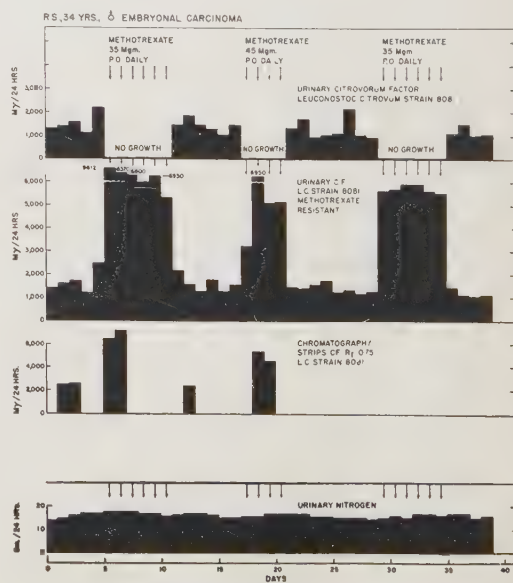


FIG. 2. Patient R. S. urinary excretion of CF and nitrogen.

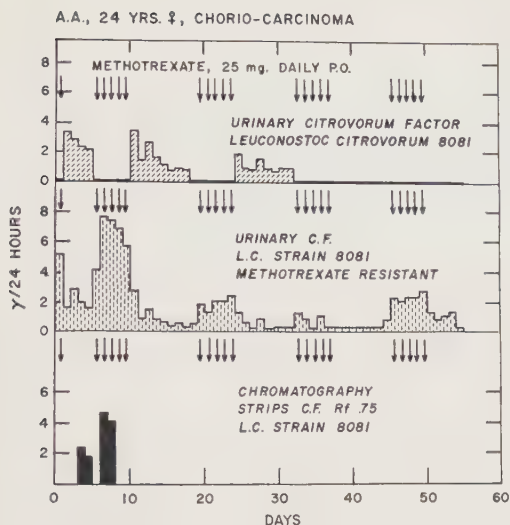


FIG. 3. Patient A. A. urinary excretion of CF.

illustrated in Fig. 3. A similar increase of urinary CF activity was again obtained on the days when methotrexate was given. However, the increase seemed to diminish toward the end of each course and the rise on subsequent courses was less prominent than the preceding ones. Tumor regression was obtained in her case. The studies on patient G.S., a 30-year-old man with metastatic choriocarcinoma to lungs and abdomen are illustrated in Fig. 4. The data, which are similar to those obtained in patient R.S., show an increase in urinary CF activity upon methotrexate administration.

Discussion. In 56 observations on 3 patients, a rise in excretion of urinary CF activity occurred when the patients received methotrexate. Using *L. citrovorum* #8081, this increase was masked, but could be measured after the separation of the material with CF activity from the other materials in the urine. This increase in urinary CF activity could be measured using the *L. citrovorum* #8081/A test organism without a preliminary separation of this material (Fig. 2 and 3). Thus, *L. citrovorum* #8081/A is a more valid micro-organism for the measurement of CF activity of materials containing methotrexate.

The increase in urinary CF activity occurred both in the presence and the absence of a response to therapy. Thus, there was not a correlation of response and increase in

urinary CF activity. This would also suggest that the urinary CF was not coming from the resolution of tumor tissue. It was thought, probably, not to be coming from the breakdown of normal tissue either, as the result of drug toxicity, since the catabolic effect of the drug was minimal as indicated by the essentially constant daily nitrogen excretion.

Pharmaceutical preparations of methotrexate contain 1 to 2 or more per cent contaminant of folic acid which might contribute to an increased urinary CF activity. If this were occurring one might expect to see the same amount of increased urinary CF activity after the same dose each time it was administered. Instead, the same amount was not excreted by each subject receiving the same amount and less was excreted after successive doses.

Nichol(2) has concluded from observations in animal and *Streptococcus faecalis* that the folic acid antagonists have at least 2 actions, one on the conversion of folic acid to CF and one on the utilization of CF after it is formed. If utilization of CF is blocked, then one might expect a part of this preformed, unusable CF to be excreted in the urine. Nichol(2) when measuring urinary CF after aminopterin administration to rats has observed such an increased excretion in some of the rats, and has interpreted this as possible displacement of CF. Swenseid(6) has examined the urine

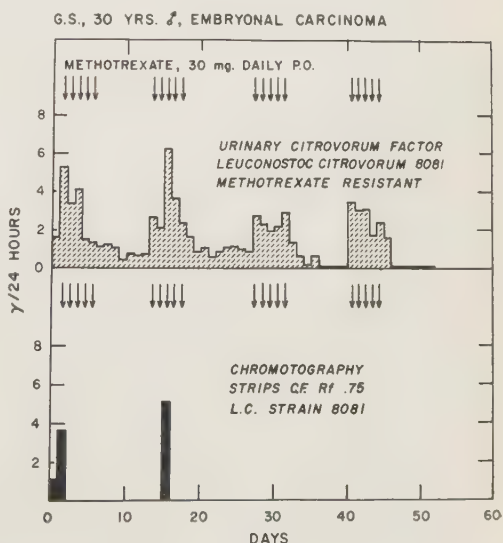


FIG. 4. Patient G. S. urinary excretion of CF.

of leukemic patients upon giving test doses of folic acid 6-10 days after aminopterin administration and has observed an increase in the excretion of a material that would support the growth of *Streptococcus faecalis*. She has interpreted her data to suggest a displacement by the antagonist. Thus, in agreement with Swenseid and Nichol, the present direct finding of a 3-5 fold increase in urinary CF activity in man after methotrexate administration probably reflects a displacement of CF by the drug.

Summary. *L. citrovorum* #8081/A of Hutchinson's laboratory has been shown to be an appropriate microorganism for measurement of urinary CF activity under the experimental conditions described. A 3-5 fold increase of urinary CF activity was repeatedly observed in subjects receiving large doses of methotrexate (Amethopterin). The possible

sources of this increased urinary CF activity were discussed.

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Effects of DON (6-Diazo-5-Oxo-L-Norleucine) and Azaserine on the Sand-Dollar Embryo.* (23639)

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The antimetabolites, DON (6-diazo-5-oxo-L-norleucine) and azaserine (0-diazo-acetyl-L-serine), are highly toxic, and often produce developmental abnormalities in chick(1,2), rat(3-5) and dog(6) embryos. It was of interest to examine the effects of these agents on echinoderm embryos, since there are many studies on the biochemical events associated with their development(7-9). Sand-dollar embryos (*Echinarchius parma*) are available in quantity during July and August in Salisbury Cove, Me., and their development, while slower, parallels that of the more extensively studied sea-urchin embryo.

Materials and methods. Eggs and sperm

were obtained from sand-dollars by the KCl-injection method(10). The eggs were fertilized in a finger-bowl by adding 5 drops of dilute sperm. In most experiments 200 to 500 fertilized eggs were then transferred to compartments of plastic ice-cube trays, each compartment containing 10 cc of filtered sea-water. The trays were floated on circulating fresh sea-water, so that the embryos were kept at the temperature of the sea, 13-15°C. The embryos were examined under a dissecting microscope at regular intervals up to 72 hours after fertilization. At 36 hours, 5 cc of fresh sea-water was added to each compartment to replenish the oxygen supply. The chemicals used were dissolved in filtered sea-water; appropriate dilutions were made in sea-water from stock solutions which were prepared

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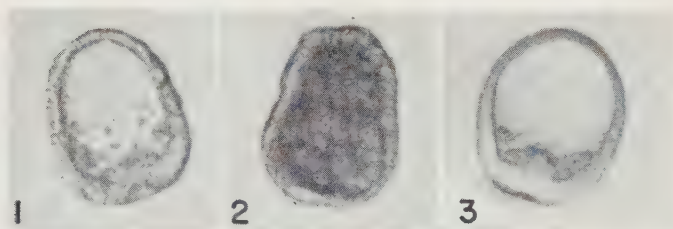


FIG. 1. Sand-dollar embryos, 22 hr after fertilization: 1. Control, early gastrulation. 2. Embryo treated with 8 m γ DON/10 cc blocked in late blastula stage. Blastula cavity contains dissociated cells, cytolysing. 3. Embryo, treated with 64 m γ DON and 32 γ inosine/10 cc sea-water. Apparently normal early gastrula.

each day. The sea-water was warmed in order to dissolve the poorly soluble compounds. The drugs used were DON, azaserine, adenine, adenosine, yeast adenylic acid, guanine, guanosine and sodium guanylate, xanthine, hypoxanthine, inosine, adenine 1-N oxide (11), DPN, ATP, AIC (4-amino-5-imidazole-carboxamide), glutamic acid, cytosine, thymine and uracil. DON was the principal agent used, and several experiments for purposes of comparison were performed with azaserine. The antimetabolites were added to the sand-dollar embryos within 1 to 2 hours after fertilization, and the chemicals tested for protective activity were added 1 to 2 hours later, unless stated otherwise. The drugs were added in a volume of 0.25 cc of sea-water in most cases. The concentration of the test chemicals to which the embryos were exposed is expressed as milligramma (10^{-9} g) or gamma (10^{-6} g)/10 cc of sea-water. Most experiments were performed at least 2 times, and many of the experiments, initially run in the summer of 1956, were repeated in 1957.

Results. *Normal development of the sand-dollar embryo.* The sequence of development of *Echinarchius parma* has not received the detailed attention accorded the sea-urchin egg (9). In this study the embryos usually were examined at 8-10 hours, 22-24 hours, 46-48 hours and 72 hours after fertilization. Cleavage begins within 1 to 2 hours following fertilization; at 2½ hours the embryos are usually in the 8-cell stage. At 8 to 10 hours a definite blastula has formed, movement begins around 14 hours, and the embryo hatches at 14 to 16 hours. At 22 hours early gastrulation has begun (Fig. 1-1), at 30 to 36 hours

the gut has almost completely formed, at 46 hours the *pluteus* stage is beginning, and at 72 hours well-formed plutei are present. These events proceeded consistently in the great majority of untreated embryos.

I. Effects of DON on Development. a) *DON added shortly after fertilization.* One hundred to 1,000,000 milligramma of DON/10 cc of sea-water. These doses had no important effect on cleavage, although slight retardation was noted at the highest dose. Ten hours after fertilization apparently normal blastulae were present, and at 14 hours these hatched and began to rotate. At the highest doses cytolysis began at this time, and at 22 hours the blastulae were degenerating. Even at the lowest dose, the embryo did not develop beyond the blastula stage, although some assumed a pointed appearance, suggesting early gastrulation. In some cases swimming persisted until 48 hours before the embryo disintegrated. The invariable effect at 100 milligramma/10 cc and higher doses was apparently normal development to the blastula stage, and then cytolysis and degeneration.

Sixteen to 64 milligramma/10 cc sea-water. Eight separate experiments in this dose range gave consistent results. The embryos proceeded to the blastula stage, and many were interrupted and disintegrated in the next 24 hours. At the lowest doses occasionally the embryos assumed an elongated appearance, and gastrulation began, but the mesenchyme was disorganized, filled the early gastrula cavity, and the embryo finally degenerated. In some cases the degenerating embryos continued to rotate for 48 hours, and the skeleton formed. The minimum concentration of

TABLE I. Effects of Various Doses of DON on Development.

| Dose, mg/10 cc | Total No. exp. | —No. experiments showing— | | |
|-------------------|----------------------|---|---|---|
| | | Inter- ruption in gas- trulation | Gastrula- tion, but short- armed plutei | Retarda- tion in de- velopment of plutei |
| 8 | 10 | 7 | 2 | 1 |
| 4 | 10 | 3 | 5 | 2 |
| 2 | 10 | 2 | 2 | 6 |
| 1 | 3 | 0 | 2 | 1 |

DON invariably producing a developmental block in the blastula or early gastrula stages was 16 milligramma/10 cc of sea-water (9.3×10^{-9} M).

One to 8 milligramma/10 cc sea-water. Doses of 1 to 8 milligramma of DON/10 cc sea-water produced definite retardation of development (Fig. 1-2) but the results were not as consistent as those seen at 16 milligramma/10 cc (Table I). The dose calculated to produce consistent developmental retardation at the early gastrula stage in at least 50% of the experiments is in the range of 2 to 4 milligramma/10 cc of sea-water (1.17 - 2.34×10^{-9} M).

b) *DON added at various times after fertilization.* In 2 separate experiments DON was added at 3, 7, 12 and 20 hours after fertilization. DON added up to 7 hours, exhibited unimpaired activity in blocking gastrulation. When added at 12 hours, 8 milligramma/10 cc still produced a definite block in gastrulation, but the embryos survived longer than the embryos treated earlier with DON. DON appeared to lose much of its effect on gastrulation when added at 20 hours. At doses of 64 milligramma/10 cc and higher, gastrulation proceeded to completion, but development stopped in the early pluteus stage and cytolysis occurred. At doses below 32 milligramma/10 cc pluteus formation was retarded and mixed cultures of blocked and advanced plutei were found. When DON was added to swimming plutei at 72 hours, 102,400 milligramma/10 cc of sea-water did not appear to interfere with their activity during the next 24 hours.

c) *DON added prior to fertilization.* Sand-dollar eggs and sperm were placed in separate

dishes of sea-water containing DON, 102,400 milligramma/10 cc. After 1 hour exposure the eggs and sperm were mixed in separate dishes containing the same concentration of DON in the following combinations: 1) treated eggs, normal sperm, 2) normal eggs, treated sperm, 3) treated eggs, treated sperm, and 4) normal eggs and normal sperm. In all dishes fertilization, cleavage and blastula formation proceeded in an apparently normal manner. The dishes treated with DON were all blocked in the blastula stage, the controls developed normally. It is concluded that DON does not interfere with the function of the sperm or egg in the process of fertilization, nor the subsequent cleavage.

d) *DON washed from eggs at various periods after exposure.* Fertilized eggs were placed in 50 cc of sea-water containing 320 milligramma DON (64 milligramma/10 cc). At 10, 40, 90, 165, 315, 450 and 690 minutes after exposure, 500-1000 eggs in 1 cc of fluid were removed and placed in 10 cc sea-water. The eggs were centrifuged lightly, and the supernatant fluid decanted so that less than 1 cc remained. Ten cc sea-water were again added, the eggs centrifuged, and the supernatant fluid decanted. The embryos were then placed in 10 cc sea-water and development allowed to proceed. The original solution had 64 milligramma/10 cc sea-water, and the washing procedures diluted it at least 1000 times; thus, the maximum concentration of DON in the final medium in which the embryo developed should have been less than 0.064 milligramma/10 cc sea-water. To demonstrate this, untreated fertilized eggs were placed in the supernatant fluid from the last washings; development proceeded normally. Control, washed and centrifuged embryos also showed normal development.

The results on the washed eggs were as follows: washed at 10 min.—normal plutei developed at 72 hr (complete protection). 40 min.—normal plutei developed at 72 hr (complete protection). 90 min.—at 72 hr blocked gastrula and plutei (moderate protection). 165 min.—blocked gastrula and plutei (moderate protection). 315 min.—mostly blastulae and blocked gastrula, a few early plutei

TABLE II. Summary of Observations on Protection Provided by Purines, Pyrimidines and Related Agents against DON in Sand-Dollar Embryo. (Maximum conc. of protective agent tested, 128 γ /10 cc.)

| Substance tested | Protective action | Conc. DON, mγ/10 cc | | | Min effective dose protective agent, γ/10 cc |
|---------------------|-------------------|----------------------|--------|-----------------|--|
| | | Degree of protection | | | |
| | | None | Slight | Almost complete | |
| Guanine | + | 256 | 128 | 32- 64 | 2 |
| Guanosine | + | | 32-64 | 8- 16 | * |
| Sodium guanylate | + | | 32-64 | 8- 16 | * |
| Adenine | + | 256 | 128 | 32- 64 | 16-32 |
| Adenosine | + | | 64 | 16- 32 | * |
| Yeast adenylic acid | + | | 32-64 | 16 | * |
| Xanthine | + | 256 | 64 | 16- 32 | * |
| Hypoxanthine | + | 256 | 128 | 32- 64 | 1-2 |
| Inosine | + | 512 | 256 | 64-128 | 2 |
| Adenine oxide | — | 4 | | | |
| DPN | + | | 64 | 16- 32 | 16-32*† |
| ATP | + | | 32-64 | 8- 16 | * |
| AIC | — | 2 | | | |
| Glutamic acid | — | 2 | | | |
| Cytosine | — | 2 | | | |
| Thymine | — | 2 | | | |
| Uracil | — | 2 | | | |

* Not titrated for minimum effective dose.

† Swimming movements blocked.

(poor protection). 450 min.—almost complete interruption in early gastrulation, rare plutei (poor protection). 690 min.—all died in blocked blastulae stage. It thus appears that if the DON is removed up to 40 minutes after exposure complete protection occurs; from 90-165 minutes there is moderate protection; 5 to 7½ hours, definite but poor protection and after 11½ hours, no protection was observed.

II. *Agents protecting against the toxicity of DON.* a) *Protective effects of various agents.* In protection studies, usually the protective agent was given at 32 γ /10 cc, and DON at doses of 128, 64, 32, 16 and 8 milligram/10 cc of sea-water; thus, the dosage ratios are in the range of 250-4000:1. These experiments were repeated a number of times, with several variations in dosage, and the following consistent results were obtained (Table II). The most active protective agents were *guanine*, *inosine* and *hypoxanthine*. Guanine and inosine were tested at minimal doses, and 2-4 γ partially protected against 64-128 milligram DON/10 cc (Fig. 1-3). Increasing the dose of guanine and inosine did not protect against higher concentrations of DON. Adenine and xanthine were less active, and the ribosides and ribotides of adenine and guanine were less effective than

adenine but gave some protection against doses of 32 to 64 milligram of DON. DPN and ATP also gave definite protection. It is of interest that DPN paralyzed the swimming movements of the embryos, and although development proceeds for a time, the plutei were clumped at the bottom of the dish. When DPN is added to untreated motile gastrulae or plutei, 32 γ /10 cc stopped movement within 1 minute. Adenine oxide, AIC, glutamic acid, cytosine, thymine and uracil gave no appreciable protection against DON. At the doses used, the agents examined for protective activity, with the exception of DPN, appeared to have no adverse effect on the development of the embryo.

b) *Protective effects of inosine when given at various periods after DON.* Inosine, 32 γ , was added to embryos exposed to doses of DON ranging from 16 to 4096 milligram/10 cc. DON was added 1 hour after fertilization, and inosine was given either 20 minutes before or after DON. Inosine had similar protective activity when given before or after DON; there was little protection at 256, slight at 128, and complete protection at 64 milligram DON/10 cc. Embryos, exposed to various doses of DON (64, 32, 16 milligram/10 cc) shortly after fertilization, were treated subsequently (3½, 6, 9, 12 and 20 hours after ferti-

lization) with inosine (32 γ /10 cc). Inosine provided protection against DON when added up to 12 hr. Protection was poor at 20 hr, where evidence of blastula interruption was present before inosine was added. Embryos exposed to lower doses of DON (16 milligramma/10 cc) and treated with inosine at 20 hours sometimes gastrulated, but were blocked in the early pluteus stage, suggesting that slight protection was possible even at 20 hours.

III. *Azaserine (Diazo-L-Serine)*. Azaserine, an analogue of DON, was compared with DON for its effects on the sand-dollar embryo. Interruption in the blastula stage was produced by 500 milligramma/10 cc, more advanced development with interruption in gastrulation occurred at 200-250 milligramma/10 cc, and mixed development, blocked gastrulae and plutei at 100-128; normal plutei were found among embryos treated at 64 milligramma. Thus, the effects produced by 500, 256 and 128 milligramma of azaserine/10 cc of sea-water were equivalent to those of 16, 8 and 4 milligramma of DON, an activity ratio of approximately 32:1. In protection studies guanine at 32 γ /10 cc gave slight protection at 2560 to 5120 milligramma of azaserine/10 cc; definite protection at 1280, and complete at 640. Adenine was less active. The protection ratios are thus not related to the amount of the azaserine or DON used, but to the multiples of the minimum effective doses of DON and azaserine.

Discussion. The effects of DON and azaserine on the sand-dollar embryo are apparently similar, but DON is approximately 32 times more active by weight. The data suggest that DON is blocking a system in the embryo which appears or begins to function some hours after fertilization, and is uniquely susceptible to the action of DON. It further appears that the protective agents are acting in a non-competitive manner, replacing an essential substance whose presence or function has been blocked by DON.

The protective action of the purines suggests that DON and azaserine interfere with purine synthesis in the sand-dollar embryo; this has already been demonstrated in bacteria(12,13), tumors, leukemic and normal

tissues(14,15) and pigeon liver system(16, 17). In the pigeon liver system, this has been shown to be due to the specific inhibition of one reaction in purine synthesis, the conversion of formylglycinamide ribotide (FGAR) to formylglycinamide ribotide (FGAM). Glutamine is the natural substrate of this reaction, and the antimetabolites inactivate the enzyme system involved in transferring an amino group to FGAR to form FGAM. Glutamine added before azaserine or DON *in vitro* will protect this enzyme to some extent, but glutamine is of no protective value when added after the antimetabolites(17). There is evidence that higher concentrations of azaserine and DON affect other systems(15,17), presumably by interfering with other reactions involving glutamine. DON is about 20 to 40 times as active by weight as azaserine *in vivo*(2,15) and *in vitro*(17); differences of the same magnitude as those found in the sand-dollar studies. These antimetabolites appear to be considerably more active, however, in inhibiting sand-dollar development. Thus, the amount of DON inhibiting FGAR to FGAM in the pigeon liver system (estimated at 7.5×10^{-5} M)(17) and the concentrations of azaserine inhibiting bacterial growth (6×10^{-6} M)(13) may be contrasted with the concentration of DON blocking the sand-dollar embryo (1.75×10^{-9} M).

While the locus of action of azaserine and DON is well-defined, there is evidence that the effects of DON, or the activity of protective substances are not the same in several biological systems. Thus, for example AIC and adenine protect the chick embryo to some extent against the teratogenic effect of DON, and guanine is ineffective(2), whereas AIC does not protect the sand-dollar embryo, and guanine is more protective than adenine against DON. In contrast to the marked sensitivity of the sand-dollar embryo to azaserine, the frog embryo is reported to be unaffected when exposed to a 0.25% solution of azaserine (25,000,000 milligramma/10 cc) over a 12-day period(18).

Why does the interruption of *de novo* synthesis of purines by DON and azaserine fail to produce a developmental block until the blastula or early gastrula stage, when cell di-

vision is proceeding actively prior to this stage? The fertilized egg contains a large amount of RNA, and relatively little DNA. Hoff-Jorgensen and Zeuthen(19) describe cytoplasmic deoxynucleosides in high concentrations in the sea-urchin and frog egg cytoplasm, which may serve as precursors of DNA. There is good evidence that the DNA does not come from cytoplasmic RNA, but is formed independently (20,21,22). Abrams (22), on the basis of glycine C^{14} studies, states "the larger part of the DNA purines (in sea-urchin embryos) probably were derived from an endogenous precursor the nature of which is unknown at present." He estimates that 75% of DNA guanine and 88% DNA adenine are obtained from endogenous sources, the glycine label being twice as high in the DNA guanine as compared to DNA adenine, whereas the RNA purines are equally labelled. Hultin(23) has observed that the rate of incorporation of C^{14} formate into purines of the sea-urchin embryo was "rather low during the first hours of development, but rapidly increased during the early blastula stage."

If preformed endogenous purine precursors are present in the embryo, the purine deficiency produced by DON might not be manifest until the precursors were exhausted. DON and azaserine, as extraordinarily potent antimetabolites, may prove useful in analyzing the events associated with nucleic acid synthesis and embryonic development.

Summary. 1. DON and azaserine interrupt the development of sand-dollar embryo at mid-blastula and early gastrula stages. The minimum dose of DON producing consistent effects is 3 milligram/10 cc of sea-water; azaserine is approximately 1/32 as active. Large doses of DON up to 1 mg/10 cc of sea-water have no appreciable effect on fertilization, cleavage or early development. 2. Various physiological purines and derivatives will protect against the action of DON and azaserine, even when added up to 12 hours after fertilization. The most active ones are guanine, hypoxanthine and inosine. The protective action of these substances appears to be non-competitive, and they are not effective against large doses of DON and azaserine.

3: In view of the fact that DON and azaserine, acting as glutamine antagonists, apparently interrupt the *de novo* synthesis of purines, it is suggested that embryonic development in the sand-dollar is blocked at the time when DNA production, initially supplied by endogenous purine precursors, becomes dependent on *de novo* purine synthesis.

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Influence of Soybean Phosphatide on Blood Coagulation and its Use in the Thromboplastin Generation Test. (23640)

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For many years a lipid substance has been postulated to be a factor necessary for the physiological clotting of blood(1). The essential lipid component probably is a phospholipid supplied by platelets(2). In the early phases of coagulation, platelets, or certain phospholipid substitutes, interact with plasma factors and calcium to form plasma thromboplastin(3). Knowledge gained from the use of the thromboplastin generation test (4) has strengthened this concept. Several workers have found that phospholipid derived from brain(5) or from soybeans(6) will provide complete substitution for platelets in this test. It is the purpose of this report to show the effect of soybean phosphatide in certain clotting systems and to illustrate its use in a simplified thromboplastin generation test.

Materials and methods. All blood was drawn by the 2-syringe technic. Normal oxalated plasma was obtained by adding 7 ml of whole blood to 1.0 ml of 1.85% potassium oxalate solution and centrifuging the mixture 10 min. at 4,000 rpm and 4°C. Platelet-rich plasma was prepared by centrifugation at only 700 rpm for 10 min. It was kept frozen and thawed just before use. A saline or aqueous soybean phosphatide[†] emulsion was made in various concentrations (water appeared to provide a better emulsion). A 0.1% concentration was used in all tests except where specifically noted. This emulsion remained fully active for at least 3 months when stored at -26°C. Barium sulfate adsorbed plasma was provided by mixing 1 ml of oxalated plasma with 100 mg BaSO₄ for 30 min. with a magnetic stirrer and then centrifuging. For the "aged serum" reagent, blood was allowed to clot and then was incubated for 2 hours at 37°C. The resulting serum was aged an additional hour at 37°C before use and was al-

ways free from demonstrable thrombin. Crude lung thromboplastin was prepared from beef lung(7). The *thromboplastin generation test* of Biggs and Douglas(4) was modified as follows: 0.3 ml of barium sulfate adsorbed plasma (diluted 1/5), 0.3 ml aged serum (diluted 1/5), 0.3 ml of 0.1% soybean phosphatide, and 0.1 ml of 0.015 M CaCl₂ were incubated at 37°C. A stopwatch was started upon the addition of the CaCl₂. The incubation times were 2, 4, 6, 8, 10, and 12 min. Thirty seconds before each of the above times 0.1 ml of the incubation mixture was added to a clotting tube containing 0.1 ml of 0.025 M CaCl₂. At precisely each incubation time, 0.1 ml of oxalated plasma was added to the clotting tube, a second stopwatch was started, and the time required for the formation of a fibrin clot was recorded. With normal plasma, thromboplastin was generated in from 6-8 min. of incubation and substrate plasma was clotted in 10-12 sec. *Prothrombin consumption test.* Three ml of whole blood in each of 2 conical centrifuge tubes was incubated at 37°C for periods of 15 and 45 min. respectively and then centrifuged for 10 min. at 4,000 rpm at 4°C. At exactly 30 and 60 min., respectively, from the time that the blood was drawn, 0.1 ml of 2.85% sodium citrate was added to each 0.7 ml specimen of the serum. After 10 min. of further incubation residual prothrombin then was estimated by the two-stage method(7) and related to the prothrombin concentration of the plasma. The percentage of residual prothrombin in normal subjects was usually below 40% in the 30-min. specimen and below 10% in the 60-min. specimen. For *one-stage prothrombin times*, 0.1 ml of rabbit brain thromboplastin[‡] was added to 0.1 ml of oxalated plasma. The mixture then was clotted by 0.1 ml 0.025 M CaCl₂. In the determina-

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† Gliddex "O" (Glidden Co.)

‡ Permaplastin (C. W. Alban and Co.)

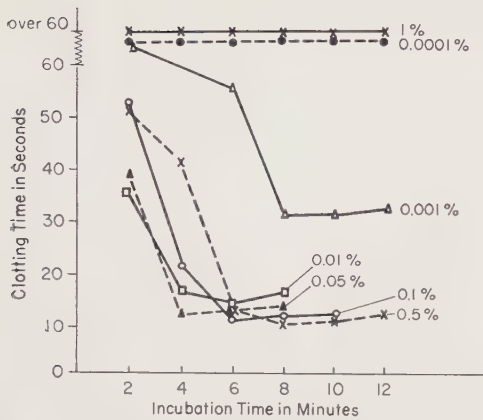


FIG. 1. Effect of soybean phosphatide concentration on thromboplastin generation test. Clotting times are those of recalcified oxalated plasma with the addition of thromboplastin generated at the indicated incubation time.

tion of the *Stypven*[§] time, 0.1 ml of *Stypven* was used as thromboplastin in the one-stage prothrombin time. The *clotting time of recalcified plasma* was ascertained by mixing 0.1 ml of oxalated plasma and 0.1 ml of normal saline, and recording the clotting time at 37°C produced by the addition of 0.1 ml of 0.025 M CaCl_2 . The same procedure was used in the “accelerated” *plasma clotting time* except that 0.1 ml of soybean phosphatide solution was used in place of saline. The *heparin tolerance test* was performed by adding 0.1 ml of varying concentrations of heparin^{||} to 0.1 ml of oxalated plasma. The mixture was then clotted at 37°C by 0.1 ml of 0.025 M CaCl_2 . The *protamine tolerance test* was performed similarly with protamine[¶] substituted for heparin. *Whole blood clotting times* were carried out by the Lee White technic. All of the above clotting assays were carried out in plain glass tubes.

Results. *The effect of phosphatide concentration on thromboplastin generation test.* Five normal subjects were studied with comparable results in each instance. Fig. 1, with the data from one subject, relates the generation of thromboplastin to various concentra-

TABLE I. Effect of Varying Concentrations of Soybean Phosphatide upon Accelerated Plasma Clotting Time.

| Phosphatide | 5% | 1% | 0.1% | 0.01% | 0.001% | 0% |
|-----------------------|-----|----|------|-------|--------|-----|
| Clotting time in sec. | 281 | 90 | 55 | 68 | 110 | 130 |

tions of phosphatide emulsion. At low concentrations (0.0001%) and at high concentrations (1%), no thromboplastin was generated. Maximal thromboplastin generation occurred when the concentration was between 0.01% and 0.1%. Saline and water dilutions of the phosphatide resulted in comparable clotting activity.

Shortening of clotting time of recalcified plasma. In over 100 normal subjects, the addition of 0.1% soybean phosphatide always shortened the clotting time of recalcified plasma when plasma was obtained after centrifugation at 4,000 rpm. The “accelerated” clotting time was from two-fifths to three-fifths of the saline control. A typical acceleration was from 135 sec. to 62 sec. Phosphatide effected considerable shortening of prolonged clotting times in patients with hemophilia or circulating anticoagulants. Phosphatide did, however, fail to alter the clotting time of previously frozen platelet-rich plasma. Such plasma already had an “accelerated” clotting time.

Table I, showing data from one of 5 normal subjects, indicates that the concentration of soybean phosphatide greatly influences the accelerated plasma clotting time. At low concentrations (0.001%), there was little acceleration of clotting; at high concentrations (5%), there was a marked anticoagulant effect. Similar results were obtained in the other 4 subjects.

Effect on the “one-stage prothrombin time” using different sources of thromboplastin. Soybean phosphatide did not shorten the clotting time when brain or lung thromboplastins were used (Table II). Only the “*Stypven*” time was shortened by the addition of the phosphatide. In this test platelet-rich plasma and soybean phosphatide behaved similarly in accelerating the *Stypven* time.

Alteration of prothrombin consumption. Fresh blood was added to tubes containing

§ *Stypven* (Russell's Viper Venom 1:10,000, Burroughs Wellcome and Co.)

|| Sodium Heparin (Abbott)

¶ Protamine Sulfate (Eli Lilly)

TABLE II. Influence of Soybean Phosphatide on "One-Stage Prothrombin Time,"*

| | Clotting time (sec.) | |
|------------------------------------|----------------------|-------------|
| | Saline control | Phosphatide |
| Rabbit brain thromboplastin | 20 | 19 |
| Heated lung | † 22 | 25 |
| Stypven (1:10,000) | 21 | 9 |
| Stypven using platelet-rich plasma | 8 | 9 |

* 0.1 ml plasma, 0.1 ml of a thromboplastin, 0.1 ml saline or phosphatide, and 0.1 ml 0.025 M CaCl₂ were added together.
† 56°C for 30 min.

0.5 ml of phosphatide emulsion and prothrombin consumption was measured after 30 and 60 minutes of incubation. The results with and without phosphatide were compared in 3 normal subjects. A typical experiment is shown in Table III (A). Phosphatide apparently accelerated the prothrombin consumption since little residual prothrombin remained even at 30 min. In 4 patients with prolonged prothrombin consumption due to thrombocytopenia (platelet counts below 10,000 per cu mm), phosphatide completely corrected the abnormality. The results obtained with one patient are shown in Table III (B). The faulty clot retraction of blood from thrombocytopenic patients, however, was not altered.

Whole blood clotting time. Whole blood clotting times always were shortened by prior rinsing of the clotting tubes with the 0.1% phosphatide emulsion. Average results from 5 subjects were as follows:

| | |
|----------------|-----------|
| Saline control | 9.24 min. |
| Phosphatide | 5.0 min. |

Anti-heparin and anti-protamine action. When increasing amounts of heparin were added to normal oxalated plasma, the recalcified clotting time was lengthened progressively. Fig. 2 shows a typical normal heparin

TABLE III. Effect of Phosphatide upon Prothrombin Consumption.

| | Residual prothrombin, % | | | |
|-----------------------------|-------------------------|-----|------------------|-----|
| | Control | | With phosphatide | |
| | 30' | 60' | 30' | 60' |
| A. Normal patient | 31 | 7 | 6 | 6 |
| B. Thrombocytopenic patient | 83 | 49 | 1 | 1 |

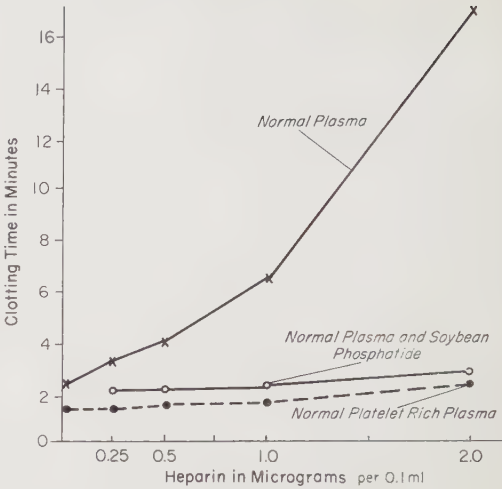


FIG. 2. Influence of soybean phosphatide and platelets upon heparin tolerance test. Clotting mixture consisted of 0.1 ml of oxalated plasma, 0.1 ml of heparin, 0.1 ml of 0.025 M CaCl₂ with and without 0.1 ml of 0.1% phosphatide.

tolerance curve and the anti-heparin effect of phosphatide. Soybean phosphatide nullified the typical heparin effect in each of the 5 subjects studied. This anti-heparin action is similar to that of platelet-rich plasma. Soybean phosphatide exhibited similar anti-heparin effects (Table IV) following administration of intravenous heparin in a dog. The heparin-induced effects in whole blood clotting time, plasma clotting time, Stypven time, and prothrombin consumption were abolished by addition of phosphatide. As expected, intravenous heparin changed greatly the *in vitro* heparin tolerance test, e.g., the clotting time with 2.0 μg of heparin increased from 13 to 31 min. The addition of soybean phosphatide nullified this change and reduced the clotting time to 2.6 min. Protamine behaved as an anticoagulant when added to normal human plasma in concentrations from 0.25 to 2.0 μg per ml. In a clotting system analogous to that used in the heparin tolerance test 2.0 μg of protamine lengthened the clotting time from 120 sec. to 240 sec. The use of phosphatide together with this concentration of protamine reduced the clotting time to 105 sec.

Partial thromboplastic activity. Like human brain cephalin, soybean phosphatide is a partial thromboplastin. When it is used in

TABLE IV. Effect of Soybean Phosphatide on Clotting Tests before and 20 Min. after 10 mg of Intravenous Heparin in the Dog.

| | Whole blood clotting time (min.) | Plasma clotting time (sec.) | Stypven time (sec.) | Prothrombin consumption (% of residual prothrombin) | Heparin tolerance test (Times in min.) | | | |
|-----------------------|----------------------------------|-----------------------------|---------------------|---|--|------|------|------|
| | | | | | (conc. of heparin in $\mu\text{g}/0.1\text{ ml}$) | | | |
| | | | | | .25 | .5 | 1 | 2 |
| <i>Before heparin</i> | | | | | | | | |
| Control | 3 | 124 | 11.5 | 0 | 2.3 | 3.3 | 5.6 | 13.0 |
| Plus phosphatide | 3 | 44 | 3 | 0 | 1.1 | 1.2 | 1.5 | 2.6 |
| <i>After heparin</i> | | | | | | | | |
| Control | 24 | 230 | 17.3 | 70 | 6.9 | 13.0 | 18.0 | 31.0 |
| Plus phosphatide | 9 | 60 | 3 | 0 | 1.2 | 1.2 | 2.0 | 2.6 |

the thromboplastin generation test on patients with classical hemophilia and with plasma thromboplastin component (PTC) deficiency, thromboplastin is not generated (Fig. 3 and 4). Only when the system is supplied with the appropriate factors present in either normal barium sulfate adsorbed plasma (Fig. 3) or in normal serum (Fig. 4) will plasma thromboplastin be generated adequately. Soybean phosphatide proved also to be a partial thromboplastin in the 2-stage prothrombin test. Substituted for the usual lung thromboplastin, the phosphatide converted little prothrombin to thrombin.

Discussion. Soybean phosphatide contains a mixture of phosphatides: lecithin, phosphatidyl ethanolamine, phosphatidyl serine, and inositol phosphatide. Rouser and co-workers have indicated recently that only phosphatidyl ethanolamine is an accelerator of blood clotting, whereas both phosphatidyl serine and inositol phosphatide behave as inhibitors of

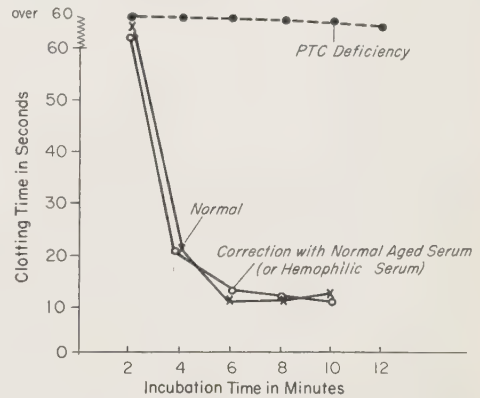


FIG. 4. Use of soybean phosphatide in thromboplastin generation test of PTC deficiency.

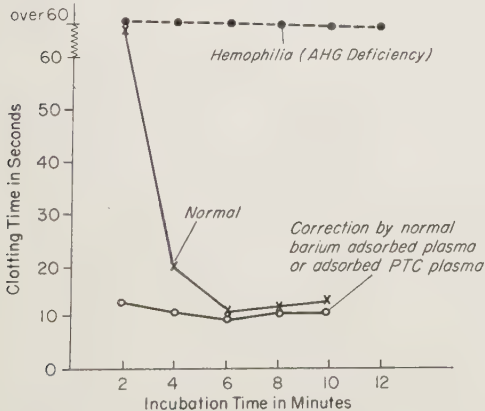


FIG. 3. Use of soybean phosphatide in thromboplastin generation test of hemophilia.

coagulation(8). Barkham obtained substantially the same results with the use of phosphatidyl ethanolamine and phosphatidyl serine, fractionated from human brain(9). Chemically pure lecithin has no activity in blood clotting systems(8). O'Brien has compared the similarities of platelets and phosphatidyl ethanolamine in blood coagulation(10). These similarities suggested to Macfarlane that the substance in platelets active in the generation of plasma thromboplastin possibly may be phosphatidyl ethanolamine(3). It therefore seems likely that a soybean phosphatide emulsion may influence blood clotting either as a lipid accelerator or as a lipid inhibitor depending on the concentration of the emulsion. White *et al.* found that concentrations of soybean phosphatide above 0.25% were inhibitory in the clotting of dog plasma(11). Our results, obtained by varying the concentration of soybean phosphatide in the thromboplastin generation test, have

indicated an optimal concentration range for maximal thromboplastin generation. High concentrations were inhibitory. Klein *et al.* had noted the same phenomena when the concentration of platelets was varied in the thromboplastin generation test(12). Concentrations of platelets above 1,000,000 per cu mm were inhibitory; concentrations below 50,000 per cu mm had little activity. Platelets at the equivalent physiological concentration had maximal effect in generating thromboplastin. Likewise, the effect of varying concentrations of platelets on the clotting time of recalcified plasma(13) is similar to our results indicated in Table II with differing concentrations of soybean phosphatide.

Soybean phosphatide failed to shorten the one-stage prothrombin time when either brain or lung thromboplastin was employed. Both of these moieties contain phosphatides(14). On the other hand, the "Stypven" time was shortened greatly by phosphatide and by platelet-rich plasma. The clotting activity of the Russell viper venom protein has been shown to be influenced greatly by either phosphatides or platelets(3).

White previously had reported that soybean phosphatide, administered intravenously, restored to normal the deficient prothrombin consumption in thrombocytopenic dogs(11). Our results show that *in vitro* addition of soybean phosphatide to blood of thrombocytopenic patients will correct their abnormal prothrombin consumption. Even in normal blood the prothrombin consumption is considerably accelerated. This presumably occurs because relatively large amounts of the phosphatide thromboplastin factor are supplied at the instant blood begins to clot. When blood is allowed to clot in a plain dry glass tube, the gradual breakup of platelets probably provides the phosphatide thromboplastin factor at a much slower rate. The shortening of whole blood clotting time by phosphatide may be due to the same reason. Platelet-rich plasma thawed from the frozen state and soybean phosphatide each accelerated the clotting time of recalcified plasma. The use of these reagents together did not have a synergistic effect.

The lipid brain extract used by certain

workers(5,15,16) as a substitute for platelets would appear to be analogous in thromboplastin activity to the "cephalin" preparations of earlier investigators. It is now known that cephalin is a mixture of the phosphatides; namely, phosphatidyl ethanolamine, phosphatidyl serine, and inositol phosphatide(17). Crude brain cephalin inhibited coagulation in high concentrations but in lower concentrations had clotting accelerating effects(15) similar to those described above for soybean phosphatide. Both Wolf and Rapaport have found that cephalin from brain does not have anti-heparin properties found in platelets(16, 18). The results in Fig. 2 and Table IV, however, indicate that soybean phosphatide has definite anti-heparin properties. The phosphatide abolished the usual anticoagulant effects of heparin in both human and dog plasma. Platelet-rich plasma behaved similarly in the heparin tolerance test. Protamine, a heparin ionic antagonist, is known to be an anticoagulant *in vitro*(19). Soybean phosphatide then appears to have the capacity of opposing the anticoagulant action of both protamine, a basic protein, and heparin, an acidic substance.

The use of soybean phosphatide as a platelet substitute has simplified the thromboplastin generation test in the study of patients with hemorrhagic disorders. It might appear that the use of a platelet suspension in this test would be more physiological. However, the preparation of platelets by freezing and thawing(20) or by repeated centrifugation and stirring with a wooden applicator stick(4) is hardly physiological. Intact platelets as such would seem to have little value in the thromboplastin generation test. The use of soybean phosphatide in this test insures a convenient uniform source of an apparently essential component. The siliconizing of glassware and needles is not necessary. Platelet defects such as occur in thrombasthenia or thrombocytopenia can be detected readily by the prothrombin consumption test. The complete correction of any abnormal prothrombin consumption by the *in vitro* use of soybean phosphatide would appear to confirm the diagnosis of such a platelet deficiency.

Summary. By its use in a variety of blood

clotting systems, soybean phosphatide has been shown to act as a partial thromboplastin in blood coagulation. *In vitro*, this substance displayed anti-heparin activity in human and dog blood and anti-protamine activity in human plasma. A simplified thromboplastin generation test is described for the study of hemorrhagic disorders. Soybean phosphatide serves as a complete substitute for platelets in this test.

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Inclusion Bodies of the Vaccinia Virus.* (23641)

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Kato *et al.*(1) presented evidence that ectromelia virus can propagate in the Ehrlich ascites tumor and in the Yoshida sarcoma cells, forming inclusion bodies of two kinds. Kamahora *et al.*(2) found that the fowl pox virus also produces 2 kinds of inclusion bodies, one of which corresponds to the Bollinger body, and the other, newly discovered by them, is similar to the "B" type body of the ectromelia virus. They stated that the "B" type body plays the chief role in the process of multiplication of these viruses, and apparently is the site providing the nucleoproteins needed for the synthesis of the viral elementary bodies. The Marchal and Bol-

linger bodies, which had been considered by other investigators as virus colonies, are merely reservoirs of mature virus. They noticed that the "B" type bodies are similar to the Guarnieri bodies observed in Giemsa-stained smear preparations of tissue infected with vaccinia virus.

In the present study, the vaccinia virus inclusion body was investigated in an attempt to establish the nature of the Guarnieri body.

Materials and methods. The refined vaccinia virus vaccine made in the Research Institute for Microbial Diseases, Osaka University, was used throughout this investigation. Adult rabbits and incubating eggs (12-day-old) were used as hosts of the virus. In the case of the rabbits the vaccine ID₅₀ titer 6, mixed with penicillin, was pasted on the cor-

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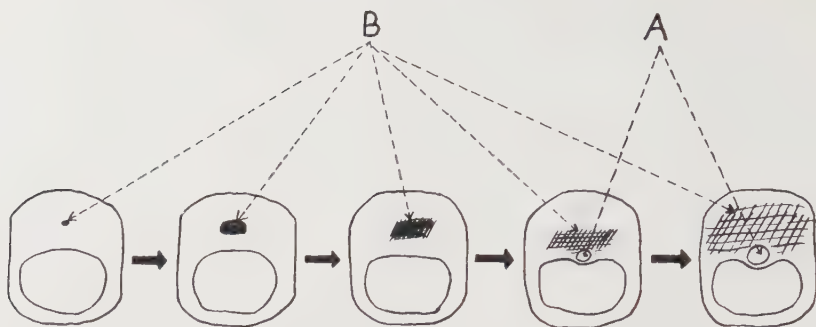


FIG. 1. Development of 2 kinds of inclusion bodies A and B. Giemsa staining.

nea and abdominal skin, which had previously been rubbed with sand paper. Scratch preparations of the surface of the cornea were made by a cataract knife at intervals of 24, 48 and 72 hours. In the incubating egg, vaccine from 2 or 3 successive inoculations was inoculated on the surface of the chorioallantoic membrane according to Burnet's method. Material of the same titer as inoculum for the rabbit cornea was diluted to 1/10 and 0.1 ml inoculated in 12-day-old eggs which were observed at 24, 48 and 72 hour intervals. The material was observed both as section and smear preparations, stained with Giemsa solution and hematoxylin-eosin and methyl blue-orange G-eosin. Feulgen reaction was carried out following Stowell's method(3), as modified by Itikawa(4) with Schiff's solution at pH 3 to give intensive reaction. PAS reaction for polysaccharide and Sudan IV staining for fat were used.

Results. Smear preparation, Giemsa staining. In the smear preparation taken 24 hours after inoculation, many structures are recognizable, such as: large homogeneous bodies; small, medium and large networks, as well as more diffuse ones over the entire cytoplasm as described by Bland and Robinow(5) (Fig. 2, A). In the smears taken at 48 and 72 hours, many types of inclusions are noticed. They gradually increase in number, especially the more diffuse ones. This process from small compact-body to large network body is clearly evidenced, although not as readily as in the case of ascitic tumor cell-ectromelia virus system. The development of vaccinia inclusion is essentially similar to the process for the "B" type inclusion body of ectromelia

virus and fowl pox virus. There are pale bluish small bodies near some of the so-called Guarnieri bodies (red) in the smear preparation made about 48 hours after infection (Fig. 2, B). Although the frequency of the appearance of these pale bluish small bodies is rather low, they always coexist with red-dened network of the Guarnieri body. They resemble the "A" type body of the ectromelia virus. For convenience, we denote the body stained red by Giemsa as "B" body and the one stained blue by Giemsa as "A" body of the vaccinia virus. The size of the "A" body is 2-3 μ in the early stage, and increases to a maximum of 5-6 μ . As a rule, there is one "A" body per cell; occasionally 2 or 3 are seen.

Cytochemical study. Feulgen reaction of the "A" type inclusion body in smear preparation is always negative. The compact and homogeneous "B" type body in the early stage always shows a positive reaction and in the more advanced stage is fainter in color. Fat and polysaccharide staining are negative in both types of inclusion. Both varieties are resistant to 1 N HCl (60° C, 10 minutes), while the "A" body is resistant to 10% trichloroacetic acid (90° C, 15 minutes). The above mentioned cytochemical character of the "B" body of vaccinia virus is identical with the character of the "B" body of ectromelia virus and fowl pox virus. The "A" body of vaccinia virus is identical with the "A" body of ectromelia virus.

Relation of "A" and "B" bodies to the Guarnieri body. We examined smear preparations using hematoxylin-eosin staining. Cells containing both "A" and "B" bodies

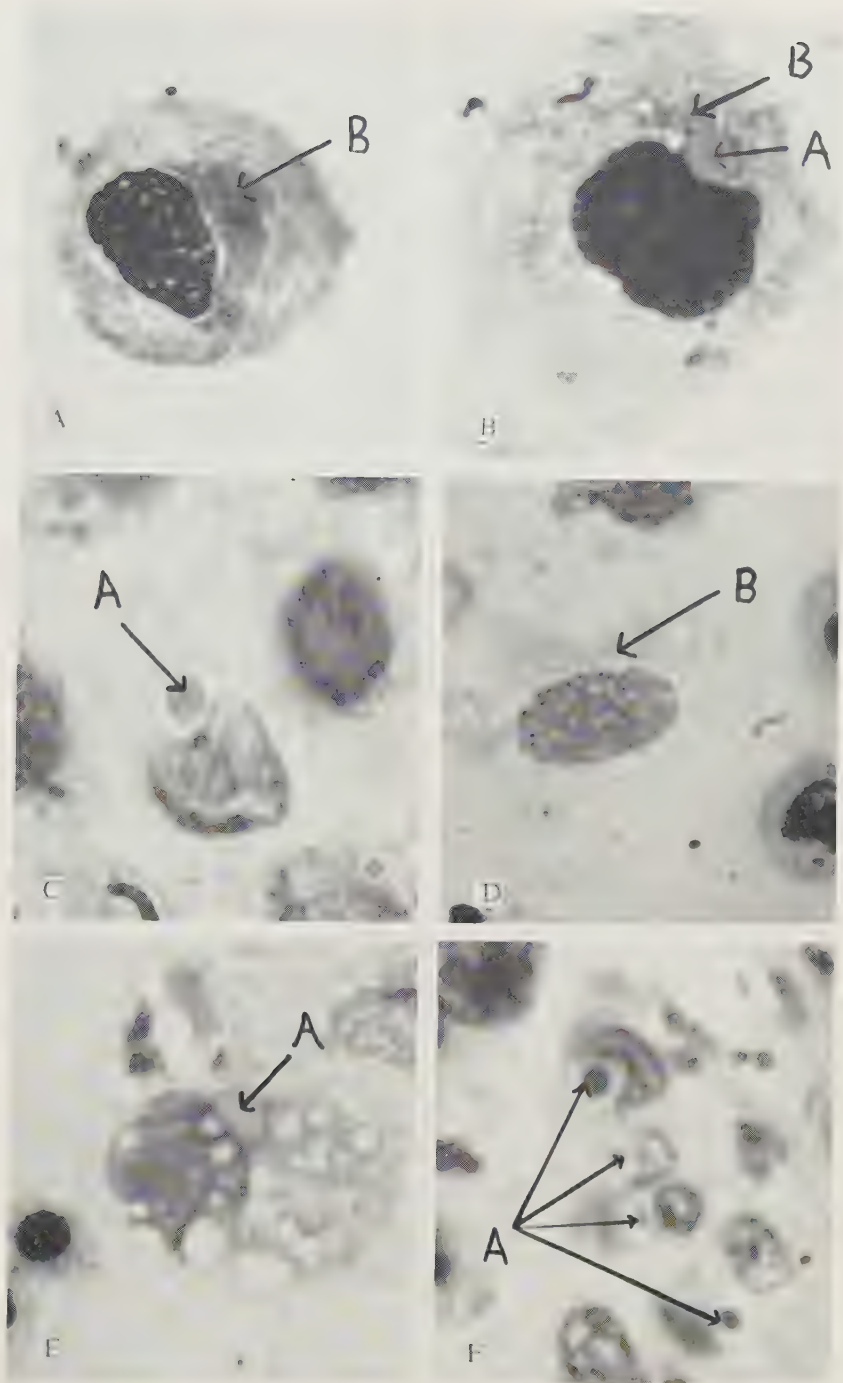


FIG. 2. (A) Large network "B" type body (red), seratch preparation, cornea of rabbit infected with vaccinia virus, 48 hr, Giemsa. $\times 900$. (B) "A" type body (blue), a diffuse "B" type body, seratch preparation, same tissue. 72 hr, Giemsa. $\times 900$. (C) "A" type body (red), section preparation, cornea of rabbit. 96 hr, hematoxylin-eosin. $\times 900$. (D) Large network "B" type body (red), seratch preparation, chorioallantoic membrane infected with vaccinia virus. 48 hr, Giemsa. $\times 900$. (E) "A" type body (blue), seratch preparation, chorioallantoic membrane infected with vaccinia virus. "B" type body (red) is diffuse. 72 hr, Giemsa. $\times 900$. (F) Many "A" type bodies (red), section preparation, skin of rabbit infected with vaccinia virus. 96 hr, hematoxylin-eosin. $\times 900$.

were stained faintly with Giemsa solution and the locations of both bodies were marked. After being decolorized completely by water and ethyl alcohol, these cells were treated with hematoxylin-eosin or eosin-orange G-methyl blue. The part corresponding to the "B" type body lost its contrast and was impossible to distinguish from the surrounding cell structure. However, "A" body took the tinge of red more strongly and sharply. This phenomenon was also observed in the ectromelia virus, a further similarity in the development of these 2 viruses. We compared simultaneously smear and section preparations using the same stage material. For instance, there were many compact "B" type inclusions seen in the smear preparation that was made from 24-hour material stained by Giemsa solution, but we were able to recognize only a few red inclusions stained by hematoxylin-eosin in the section preparation made from the same material (Fig. 2, C). The difference in the staining and appearance of the bodies from smear and section preparations, indicates that the so-called Guarnieri body in sections does not correspond with the so-called Guarnieri body in smear preparations. Actually, there appear to be 2 kinds of so-called Guarnieri bodies, although previous investigators regarded them as a single type of inclusion. Fig. 1 summarizes our findings.

Chick chorioallantoic membrane preparation. Goodpasture *et al.*(6) reported an acidophilic inclusion body in sections of this tissue stained by acid fuchsin-methylene blue, although according to Beveridge and Burnet (7) bodies resembling the Guarnieri bodies of the rabbit cornea are rarely found. We examined the ectodermal layer of the chorioallantoic membrane infected with vaccinia virus by scratch preparation. In 24-hour specimens, we could easily recognize numerous red "B" type inclusions in the cells of the ectodermal layer stained by Giemsa solution. With time, the "B" type inclusion bodies gradually increased in number and showed a diffuse reticular structure (Fig. 2, D). In the preparations made at 48 hours and 72 hours, we found small blue stained bodies ("A" type inclusions) coexisting with some of the net-work of "B" type inclusion bodies

(Fig. 2, E). The relationship between "A" and "B" type bodies, their stainability and their morphological characteristics in the smears and in sections of the chorioallantoic membrane were completely identical with those of the rabbit cornea.

Abdominal skin of rabbit preparation. The same method of inoculation was used as in the case of rabbit cornea. We obtained the same results in the Malpighian layer of the skin as in the case of the rabbit's cornea (Fig. 2 F).

Discussion. In our previous experiments (1,2), we established that there are 2 kinds of inclusions in the cell infected with the ectromelia and fowl pox viruses. Prior to our present work, it was believed that the Guarnieri body was the only inclusion in vaccinia. This inclusion was first described by Weigert and Pfeiffer, but Guarnieri's report is best known(8). Tyzzer(9), Downie(10), and others described the inclusion bodies as acidophilic in section preparations stained by hematoxylin-eosin, methyl blue-orange G-eosin staining or methylene blue-fuchsin. In 1905, James Ewing(11) proposed a new and different method of studying vaccinia inclusions. He examined the inclusion body both by smear preparation and by the Nochts-Romanovsky technic. Observing a deep red body in the cytoplasm, he named it vaccine body. During its development the body increased in size, and its reticular structure spread diffusely in the cytoplasm. At times, in the more advanced stage, he recognized a homogeneous blue staining material near the red vaccine body in smear preparation. Ewing's descriptions were extensive and he was the first to report the existence of 2 kinds of bodies. However, he assumed that this red vaccine body was the same as the vaccine body which he observed in the tissue section preparation. His epoch-making work considerably changed the method of study. In spite of Ewing's detailed description of 2 kinds of bodies, other investigators who used smear preparation and Giemsa staining almost always observed only deep red material, commonly called the Guarnieri body. Thus Bland and Robinow's description(5) of the development of the inclusion body in tissue

Ultrastructure of Viruses of Myeloblastosis and Erythroblastosis Isolated from Plasma of Leukemic Chickens.* (23642)

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Avian myeloblastosis and erythroblastosis are malignant leukemic diseases of the chicken caused by distinct though closely related viruses(1). These agents occur in the plasma of birds with the respective diseases, the virus of myeloblastosis in concentrations as high as 2×10^{12} particles per ml(2) and that of erythroblastosis at levels of 10^{10} to 10^{11} per ml(3). In morphology and size the 2 agents, examined by electron microscopy, either in the plasma or in ultracentrifugal concentrates of the plasma, are essentially identical(4). The spheroidal particles representing the respective agents vary widely in size, but average about 120 m μ in diameter(5,6). In both diseases, the particles have been identified by correlations of the results obtained with physical, chemical and biologic methods(7).

Examination of the particles by the usual technics of electron microscopy, involving metal shadowing, yields little evidence of the actual structure of the individual entities(1). Moreover, in the shadowed preparations, it was not always possible to distinguish unequivocally between virus and particles extraneous to the specific elements. In order to obtain information about the ultimate structure of the virus particles and of the relation of viral to non-viral material in concentrates, studies have been made of ultrathin sections of pellets resulting from ultracentrifugation of plasmas containing the agents.

Materials and methods. The myeloblastosis virus was the BAI strain A investigated at Duke University since 1949(1). This disease is a pure strain of myeloblastosis in which no evidence of abnormality of the red cells has been seen in stained smears of the

circulating blood. The erythroblastosis virus was strain "R" of Engelbreth-Holm(8) obtained in 1955 from Dr. Astrid Fagraeus. Host response to the virus and the pathologic characteristics of this erythroblastosis have been uniform and wholly different from those seen with myeloblastosis.

Plasmas used as source of myeloblastosis virus were selected for high virus content by adenosinetriphosphatase activity with the micro screening technic(9), cleared of cells by centrifugation and stored under crushed CO₂ ice for about 2 months. Virus was sedimented from 3 plasmas separately by ultracentrifugation at 15,000 x g for 45 minutes. Erythroblastosis virus was obtained in the same way from 4.6 ml of a single plasma from a bird with a high content, approximately 250,000 per ml³, of erythroblasts in the circulating blood. The actual virus content of the erythroblastosis plasma was unknown since this agent does not dephosphorylate adenosine triphosphate(3). The plasma had been frozen in CO₂ ice for about 6 months. Infectivity measurements have shown that both viruses are quite stable under these conditions. A series of 5 pellets of erythroblastosis virus was obtained from separate plasmas by like methods with a strain of the agent maintained at the Institut de Recherches sur le Cancer, at Villejuif. Here, also, analogous pellets were obtained from the plasmas of 12 apparently healthy chickens.

After centrifugation and removal of the plasma, the pellets were fixed immediately for 1 or 2 hours in 2% osmic acid buffered with veronal acetate at pH 7.3; dehydrated with alcohol; and embedded in methyl methacrylate by the usual procedures. Sections varying in thickness from about 250 to 500 Å were obtained with the Porter-Blum microtome. The electron micrographs were taken

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with the RCA EMU 2[†] or the Elmiskop I SIEMENS microscope.

Results. An electron micrograph of an ultrathin section obtained from a relatively large pellet derived from myeloblastosis plasma is shown in Fig. 1. The distinctive feature of the picture is the presence of circular or oval images representing cross sections of spheroidal particles. The images were strikingly similar in appearance but of variable diameter, usually about 80 m μ across. Some of the smallest, however, indicated a particle diameter of about 60 m μ , and the largest diameters were in the range of 110 m μ . A finely granular mass comprised the inner material which was of highest density in the central area. This central mass of high electron absorbing properties was about 35 to 40 m μ in diameter and comparable with the nucleoid seen in analogous pictures of other viruses. At a higher magnification (Fig. 2), the ultrastructure of the particles is more clearly visible. No typical structural organization was found in the nucleoid which appeared rather homogeneous. The external limiting membrane seemed to be single in most instances, but in some particles, it was distinctly separated into 2 layers (Fig. 2 and 3). The high degree of homogeneity of most specimens examined should be emphasized. In addition to the specific bodies described here, elements of variable size, corresponding to the debris of disintegrating blood cells, were also found in very small amounts.

In comparison with preparations of myeloblastosis virus, pellets from the plasma of chickens infected with the *erythroblastosis* virus are much smaller and contain fewer distinctive particles in electron micrographs of shadowed preparations(1,6). It was more difficult, also, to find the representative images in the ultrathin sections of the pellets. However, in the midst of variable quantities of cellular debris such as small mitochondria, ergastoplasmic lamellas with ribonucleo-protein granules, areas were found which con-

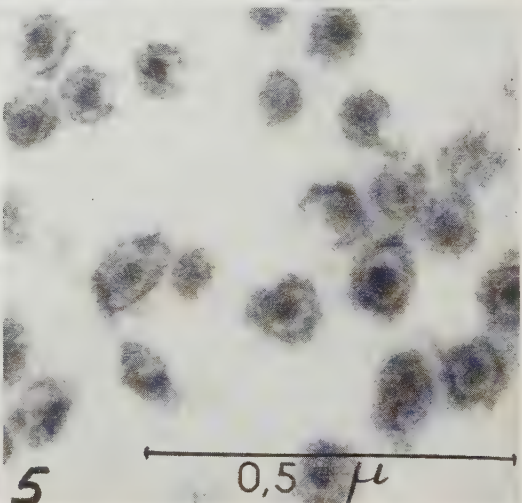
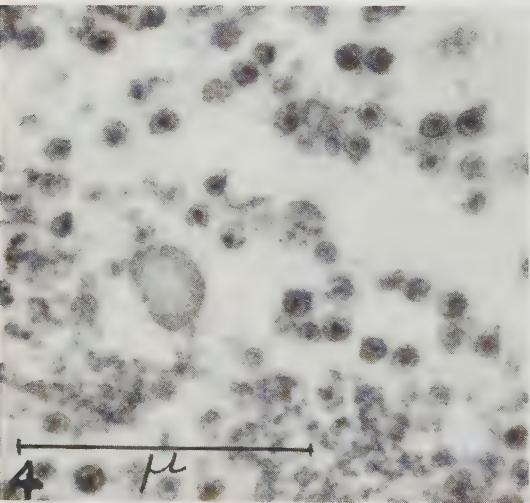
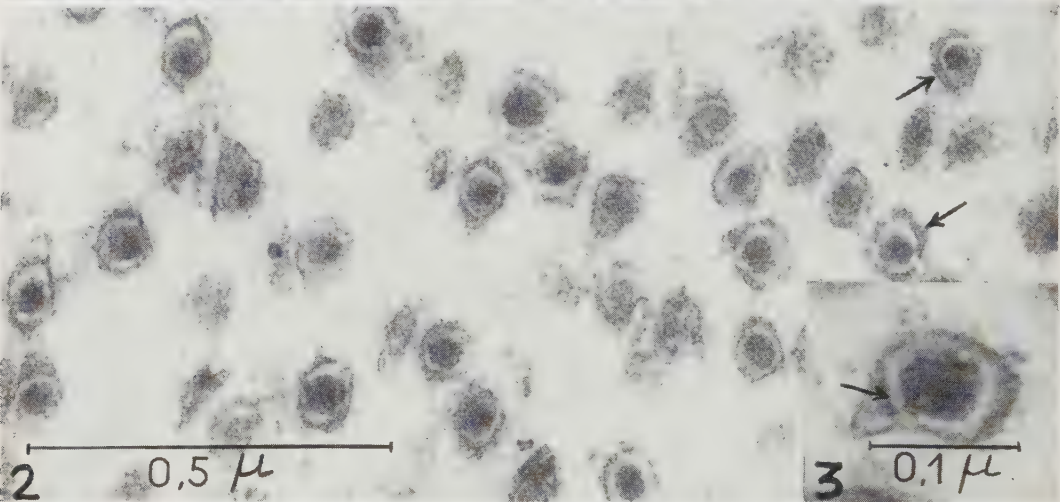
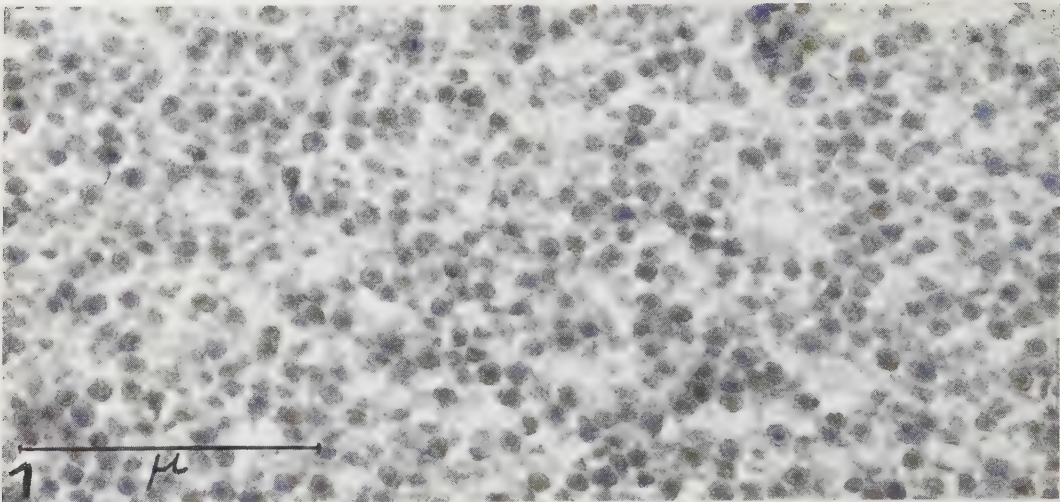
tained thousands of particles similar to those seen in the myeloblastosis pellets. Their diameters varied, also, in the range of 75-80 m μ , and they, too, were surrounded by a membrane, which appeared single at low magnification, but often was double in the pictures of high resolution (Fig. 4 and 5). The central core was 35 to 40 m μ in diameter. It can be concluded that neither with respect to size, nor to ultrastructure was there a clear morphologic distinction between the agents of erythroblastosis and myeloblastosis.

The very small pellets from plasmas of the *healthy chickens* used for these experiments did not contain the specific elements.

Discussion. The results of earlier work have served to establish the identity of the spheroidal particles occurring in the plasmas of birds with myeloblastosis and erythroblastosis as the respective specific agents of these avian leukemias. In the instance of the agent of myeloblastosis the evidence is particularly strong because of access to the high concentrations of the virus in plasma and the ready applicability of a variety of technics. Identification of the virus of erythroblastosis has been less secure, but nonetheless convincing. There has been much evidence that the concentrates from myeloblastosis plasma must be of high homogeneity. In contrast, it has been recognized that the concentration of erythroblastosis virus in the plasmas has been of a far lower order than that seen with myeloblastosis, and that, although the erythroblastosis virus was concentrated in the pellets, the proportions of the agent to extraneous substance would be relatively small. The results of the present work corroborate fully the earlier findings and greatly extend the knowledge of the structure of the particles and of the constitution of the concentrates obtained by ultracentrifugation. It is evident that the application of the technic of ultrathin sectioning constitutes an invaluable addition to the technics for establishing the content of virus preparations.

Measurements of the diameters of the images gave essentially identical values for both agents. However, there was a striking difference between the data obtained from the sections and the diameters measured in the

[†] We wish to thank Prof. D. C. Pease, of the Dept. of Anatomy, the Univ. of California, Los Angeles, who offered his hospitality to one of us and enabled him to start this work in his laboratory.



micrographs of previous studies, namely, about 120 $m\mu$ (1,6), in which the estimates were made on dehydrated virus shadowed with metal. Sizes determined in this way may have been relatively large because of flattening of the particles and the thickness of the metal. It is unlikely that the diameter indicated in the present studies is indicative of the sizes of the agents *in vivo* which, for the myeloblastosis virus, was 144 $m\mu$ measured by sedimentation analysis(5). The dehydration and embedding in preparation for thin sectioning certainly produce shrinkage of variable degree, which is very difficult to evaluate in each single case. It is nevertheless striking that the virus particles as shown in thin sections of spleen and bone marrow from chickens with erythroblastosis(10), from the Rous sarcoma(11,12), and from the Murray-Begg endothelioma(13) are of approximately the same size as that of particles outside the cells. In addition, the inner structure of the viruses described in this paper was very similar, perhaps identical, to that of the particles found in all these tumors. Slight structural differences may exist between the various agents. For instance, it is not known why, in the sections of pellets of the leukemia viruses, the two layers of the double membrane were so closely adherent and appeared separated only occasionally, whereas in the particles visible in tissue sections of erythroblastosis and other chicken tumor viruses the two membranes were clearly distinct entities(14). It seems possible that these ultrastructural differences may have been related to storage in the frozen state or to the ultracentrifugal packing and did not represent the real differences *in vivo*. There is increasing evidence that all chicken

tumor viruses belong to the same family as far as their fine structure is concerned(11,14). It is highly probable that the differences in size(11,13) or ultrastructure are due to differences in the technics applied by the various investigators. This is emphasized by recognition of immunologic interrelationships between the agents of the leukosis complex and those of the chicken tumors(1).

Summary. Ultrathin sections of the pellets formed by ultracentrifugation of plasmas from birds with myeloblastosis and erythroblastosis have been examined by electron microscopy. In the material from both diseases there were observed structures of similar appearance represented by images of round or oval shape consisting centrally of a dense core of about 40 $m\mu$ diameter. These were surrounded by a less dense material and were limited by an outer membrane-like structure with a total particle diameter of about 80 $m\mu$. In many instances the limiting membrane appeared to be double. The ultrastructure of these agents of the avian leukemic diseases was very similar, if not identical, to that of the viruses of other chicken neoplasms. No particles of these characteristics were obtained from the plasmas of apparently healthy chickens.

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FIG. 1. Electron micrograph of a relatively thick section of a pellet of myeloblastosis virus. Virus particles of similar size and structure are practically the only constituent of this sample. Magnification 40,800 \times .

FIG. 2. Ultrathin section of another pellet of myeloblastosis virus at a higher magnification. The ultrastructure of the particles with their central core ("nucleoid") and the peripheral membranes, is clearly visible. Arrows indicate regions of particles where the membrane appears duplicated. Magnification 100,000 \times .

FIG. 3. A single myeloblastosis virus particle at greater enlargement. Magnification 200,000 \times .

FIG. 4. Pellet from plasma of a chicken with erythroblastosis. Images characteristic of virus particles are clearly visible. In addition there are present disintegrating cell structures. Magnification 40,800 \times .

FIG. 5. Micrograph of the pellet from plasma of another chicken with erythroblastosis seen at higher magnification showing the "nucleoids" and outer membranes. These particles cannot be distinguished morphologically from the virus of myeloblastosis. Magnification 100,000 \times .

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Influence of *in vivo* Administration of Adrenocorticoids on Metabolism of Thymus, Liver and Tumor Tissues.* (23643)

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Prominent among approaches employed in investigations of the metabolic functions of adrenocortical hormones have been measurement of various metabolic products following administration of the hormones to live animals, and measurement of activity of specific enzymes or enzyme groups after hormone administration *in vivo* or *in vitro* (1,2). In the present studies, the hormones were administered *in vivo*, usually after adrenalectomy, and tissues were removed 4-6 hours later for *in vitro* metabolic studies. Tissues of adrenalectomized animals were employed for obtaining low-hormone control data. Because of the lability of lymphoid tissue to the action of adrenal corticoids, thymus tissue was given particular attention in anticipation of high metabolic sensitivity to cortisone preceding morphological changes (3).

Methods. Cortisone acetate (cortisone) or cortisone in combination with hydrocortisone was administered to 125-175 g male rats of the Holtzman strain or young adult male mice from the Stokely-Peterson colony 4 to 6 hours before the animals were killed and the tissues removed. The hormones, 10 mg per rat and 1 mg per mouse, were injected half by the

intraperitoneal and half by the subcutaneous routes (4). When adrenalectomized animals were employed the operations were performed 3 to 5 days previous to hormone administration to allow for recovery from the operation with negligible increase in accessory corticoid-secreting tissue. After adrenalectomy the animals received 1% NaCl and 5% glucose in the drinking water for 24 hours and 1% saline thereafter. Purina laboratory chow was fed *ad libitum*. No adrenalectomies were performed in tumor-bearing animals. Cell suspensions of thymus, liver, or Murphy-Sturm lymphosarcomas from rats, prepared by the procedure of Kit and Barron (3), and Ehrlich ascites cells, harvested from mice, were washed and pipetted into Warburg vessels containing the radioactive substrate in a side arm. The flasks were covered with serum caps through which a 95% O₂-5% CO₂ mixture was introduced by hypodermic needle. All preparative manipulations were performed in the cold, and incubations were carried out in Krebs-Ringer bicarbonate buffer (5) for 60 minutes at 37.5° with shaking. At the end of the incubation 0.2 ml of 10% KOH was added to the center well and 0.3 ml of 70% perchloric acid (PCA) to the incubation medium by hypodermic syringe and long needle. The side arm was rinsed with the acidified mixture, and the flasks were shaken for an additional hour to insure the complete libera-

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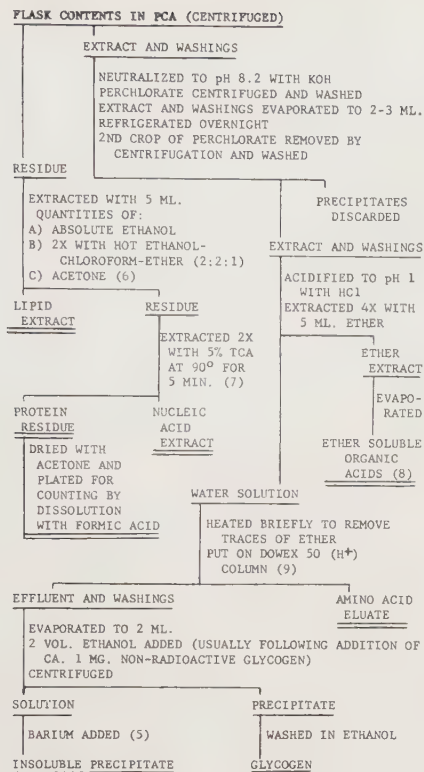


FIG. 1. Isolation of metabolic components from incubation mixtures for radioactivity assays.

tion of CO_2 . The contents of the center well were removed and assayed for C^{14}O_2 . The mixture from the main compartment was transferred to a centrifuge tube and carried through the procedures described in Fig. 1. Two dimensional descending paper chromatograms (Whatman No. 1 paper) were run employing ethyl acetate-acetic acid-water (4:4:1) *vs.* butyric acid-sodium butyrate-water (10) for the barium-ethanol insoluble residues and 80% phenol *vs.* butanol-acetic acid-water (8:2:5) for the amino acids. For the ether soluble fraction one-dimensional chromatograms were employed with a phenol-formic acid-water (79:2:19) system. Radioactive spots were located on the chromatograms by exposure of x-ray films(11), and in several instances the spots thus located were cut out, eluted and counted quantitatively. Liver glycogen was determined as glucose after treatment of the tissue with hot alkali. The counting of radioactive samples and

chemical determination of glucose have been described(4).

Results. The first experiment was designed to determine the effect of the experimental procedures on the morphology of the thymus. Male mice 9 weeks of age were given a single dose of 1 mg of cortisone as described above. The weight of thymus glands of groups of mice was determined periodically thereafter. As shown in Fig. 2, a rapid weight loss began between 6 and 9 hours and at 24 hours the gland was reduced to a third its original weight. Microscopic examination of these same glands revealed insignificant disintegration of lymphocytic cells before 24 hours. A similar study has been reported(12).

From the metabolites examined, consistent differences in the metabolism of the tissues of the adrenalectomized and adrenalectomized, hormone-treated animals were demonstrated with respect to carbon dioxide production, glycogen formation, and incorporation of radioactivity into protein. With glycine-2- C^{14} or uniformly labeled glucose as the source of radiocarbon, decreased accumulation of radioactivity into protein and carbon dioxide by thymus tissue resulted from treatment with the adrenal hormones (Table I). Similar decreases in incorporation of radioactivity into these 2 pools under the influence of hormone treatment were observed with acetate-1- C^{14}

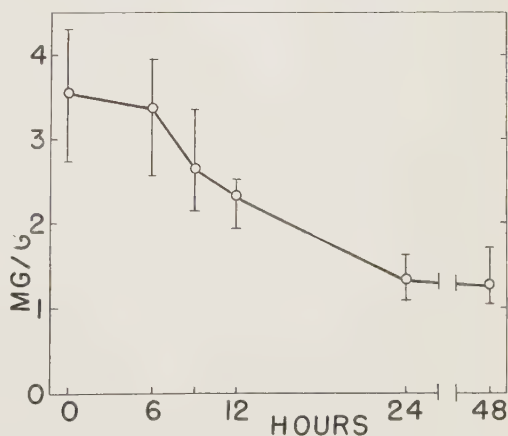


FIG. 2. Effect of a single dose of cortisone (1 mg) on wt of thymus glands. Each exp. group consisted of 4 mice while 6 mice served as controls; 3 control mice were killed at 6 hr and 3 at 24 hr. The wt of the glands are expressed as mg/g body wt, with both range and avg shown.

TABLE I. Some *In Vitro* Effects of Cortisone Administered *In Vivo*.

| Tissue, substrate and metabolic pool | Adrenalectomized (cpm/mg dry tissue)* | Adrenalectomized + cortisone |
|---|---------------------------------------|------------------------------|
| <i>Thymus, glycine</i> (.0013) †‡ | | |
| CO ₂ (97 ± 4) § | 98 ± 0 | 76 ± 1 |
| Protein (3160 ± 115) | 4105 ± 259 | 1962 ± 175 |
| Lipid (85 ± 2) | 92 ± 5 | 64 ± 5 |
| <i>Thymus, glucose</i> (.0037) | | |
| CO ₂ | 2425 ± 180 | 1493 ± 221 |
| Protein | 359 ± 26 | 180 ± 35 |
| Lipids | 94 ± 6 | 55 ± 7 |
| Nucleic acids | 74 ± 1 | 52 ± 4 |
| Ether soluble | 1265 ± 96 | 1563 ± 286 |
| Free amino acids | 2662 ± 451 | 2867 ± 222 |
| Glycogen | 41 ± 17 | 130 ± 18 |
| Ba ⁺⁺ -Ethanol Ppt. | 13900 ± 4270 | 14750 ± 3200 |
| <i>Liver, fructose</i> (.0037) | | |
| CO ₂ | 139 ± 37 | 83 ± 38 |
| Protein | 529 ± 17 | 488 ± 80 |
| Ether soluble | 1395 ± 126 | 1520 ± 224 |
| Free amino acids | 242 ± 37 | 356 ± 87 |
| Glycogen | 30 ± 3 | 45 ± 1 |
| Ba ⁺⁺ -Ethanol Ppt. | 5330 ± 1110 | 8350 ± 2650 |
| <i>Murphy-Sturm, acetate</i> (.00092) ‡ | | |
| CO ₂ | 4975 ± 65 | 2095 ± 660 |
| Protein | 617 ± 50 | 360 ± 156 |
| Nucleic acids | 67 ± 18 | 23 ± 9 |
| <i>Murphy-Sturm, glycine</i> (.0013) ‡ | | |
| CO ₂ | 14 ± 2 | 8 ± 1 |
| Protein | 476 ± 153 | 114 ± 30 |
| Lipids | 65 ± 16 | 86 ± 4 |
| Nucleic acids | 57 ± 28 | 23 ± 11 |
| <i>Ehrlich ascites, acetate</i> (.0013) ‡ | | |
| CO ₂ | 603 ± 12 | 725 ± 27 |
| Protein | 98 ± 4 | 139 ± 5 |
| Lipids | 316 ± 110 | 413 ± 36 |
| <i>Ehrlich ascites, glycine</i> (.0013) ‡ | | |
| CO ₂ | 76 ± 4 | 113 ± 7 |
| Protein | 3495 ± 195 | 3605 ± 206 |
| Lipids | 496 ± 40 | 484 ± 13 |

* Values were obtained from duplicate and triplicate determinations on tissue pools made from as many as 8 (usually 4-6) animals. All values are corrected to 3.0×10^6 c.p.m./flask and are averaged \pm stand. error of mean. Although stand. errors are included in the data, the experiments were designed to survey broad areas rather than to establish precise values.

† Radioactive substrate at molarity indicated. In addition flasks contained bicarbonate buffer, 35-65 mg dry wt of tissue, and glucose if indicated.

‡ Non-radioactive glucose added to incubation mixture at approximately physiological level, 0.0056 M.

§ Values for intact untreated controls.

|| Cortisone treatment 24 hr before sacrifice (instead of usual 4-6 hr).

or uniformly labeled fructose as substrate (not illustrated). While liver tissue also showed decreased incorporation of radioactivity into the CO₂ and protein pools in some experiments, the pattern was not consistent under the experimental conditions employed. Liver tissue, however, regularly accumulated increased radioactivity into its glycogen component under the influence of the hormone. Although predictable differences were not observed for any of the other metabolites investigated, the hormone administration was often accompanied by decreased synthetic activity in thymus tissue particularly as demonstrated by the lipid fraction.

The tissue preparations derived from Murphy-Sturm lymphosarcomas (Table I) produced radioactive CO₂ and incorporated radioactivity into protein at uniformly lower rates as a result of hormone administration. In an experiment not shown in the table, Gardner lymphosarcoma ascites cells harvested from C3H mice also exhibited an inhibition in rate of metabolism resulting from cortisone treatment. Erlich ascites cells, not derived from lymphatic tissue, were no more consistent than liver in the effects of the hormone treatment.

Autoradiograms made from the free amino acid fractions, the ether soluble fractions, and the barium-ethanol precipitates showed no qualitative or significant quantitative differences in the individual metabolites produced by the tissues from animals with or without hormone treatment.

Since the influence of the adrenal hormones upon synthesis of glycogen by liver tissue was demonstrable even during *in vitro* incubations, it was of interest to continue the studies with special attention to this constituent. Dianzani and Scuro (13) reported that 2,4-dinitrophenol (DNP) administered for 6 days to rats increased the storage of liver glycogen. Several other compounds studied by these workers were also of interest as inhibitors of various phosphate transfer reactions, most of which have been implicated in some way with the action of the adrenal hormones.

When DNP, brilliant cresyl blue (BCB), and methylene blue (MB) were administered intraperitoneally to fasting, adrenalectomized

TABLE II. Influence of Metabolic Inhibitors on Glycogen Production in Livers of Adrenalectomized Rats.

| Treatment* | Liver glycogen (mg/g fresh tissue) |
|--------------------|---------------------------------------|
| Adx | .7 \pm .1 |
| " + hormones | 6.5 \pm 1.2 |
| " + DNP | 4.1 \pm .2 |
| " + DNP + hormones | 16.0 \pm 2.6 |
| " + MB | 1.9 \pm .4 |
| " + MB + hormones | 10.7 \pm 1.6 |
| " + BCB | 1.3 \pm .4 |
| " + BCB + hormones | 16.4 \pm .2 |

* Typical experiment: 4 animals/group; animals fasted 28 hr and hormones admin. 4 hr before sacrifice; 5 mg cortisone and 5 mg hydrocortisone/rat.

rats (Table II) it was observed that DNP, given in 2-4 mg doses at the beginning of a 28-hour fast, increased the liver glycogen to 6 to 10 times the level in untreated adrenalectomized animals. MB or BCB at equimolar levels had significant but lesser effects (80-150% increases). None of the 3 inhibitors influenced the increase in glycogen content of the livers by cortisone or hydrocortisone administered 4 hours before sacrifice. Since the inhibitors are very toxic to the rats when administered during the latter part of the fast period, no evidence could be obtained to indicate that these substances increase the glycogen level in the manner of the adrenal hormones after the level has been reduced by the fast; rather they apparently prevent part of the decrease during the fasting period. Because of its toxicity Janus green B could not be studied satisfactorily at the levels employed.

Discussion. Results of the present study indicate that adrenalectomy and adrenalectomy plus adrenal cortical hormone treatment altered the metabolism of certain animal cells during a subsequent incubation *in vitro*. The metabolic changes occurred prior to the onset of rapid destruction of lymphocytes. Therefore these changes reflect a true metabolic response to cortisone rather than the result of altered tissue structure due to prolonged cortisone action.

The depression in protein synthesis (or build-up of radioactivity into protein) by the adrenal hormones in thymus tissue or tumors

of lymphatic origin may be a manifestation of the phenomenon observed by Roberts(14) in which cortical extract or adrenocorticotropin enhanced the release to the medium of protein by incubating tissues. Kit and Barron(3), who also utilized metabolizing cell suspensions but added the cortical hormones *in vitro*, reported that respiration of lymphatic cells and incorporation of C^{14} into protein from acetate, glycine, or phenylalanine was inhibited, but incorporation of C^{14} into lymphosarcoma cells was depressed only by higher hormone levels. In the present study, in which the amounts of hormones administered to the whole animals made it unlikely that quantities of the hormones comparable to those employed by Kit and Barron reached the tissues taken for incubation, similar or larger inhibitory effects on $C^{14}O_2$ production and incorporation of radioactivity into protein resulted.

The accumulation of glycogen observed in the liver may result from depression of processes accounting for the utilization or turnover of hepatic glycogen. Such a possibility is substantiated by the series of compounds such as DNP which, in spite of their inhibitory effects, increased the glycogen level in livers of fasting rats. The incidental observation of the consistently high metabolic rate of thymus tissue compared to liver marks this tissue as one deserving more study. Also to be noted is the difference in influence of adrenal hormones on the various normal tissues of the body. This variability in response indicates that a variety of tissues should be studied in investigating the basic action of the hormones.

Summary. In cell suspensions of thymus or lymphatic tumor tissues from rats treated 4 to 24 hours previously with cortisone or hydrocortisone consistent decreases in $C^{14}O_2$ production and in incorporation of C^{14} into tissue protein were demonstrated whether incubations were conducted in the presence of uniformly labeled glucose or fructose, acetate- C^{14} or glycine- C^{14} . Liver tissue exhibited increased *in vitro* accumulation of C^{14} into glycogen after hormone treatment. A variety of other metabolic pools showed no difference in total C^{14} accumulation or distribution re-

sulting from hormone administration. Methods of analysis for a wide spectrum of metabolites from tissue incubates are outlined. Dinitrophenol, methylene blue, and brilliant cresyl blue increased the level of hepatic glycogen in rats when administered at the beginning of a 28-hour fast period. These increments are in addition to the increase caused by the cortical hormones and may represent a failure to utilize the glycogen during the fast.

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Hyperlipoproteinemia and Cholesterol Deposition at the Arteries of I^{131} -Treated Dogs.* (23644)

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Steiner, Kendall and Bevans(1) confirmed and extended their original observation, that thiouracil administration to cholesterol-fed dogs will induce atherosclerosis that is distributionally and microscopically similar to the human disease. These authors pointed out that thiouracil feeding alone, for a 14-15 month period, resulted in an average blood cholesterol concentration of 394 mg % (range 176-594) and failed to cause arterial lesions. The appearance of early atherosclerotic plaques was noted, however, after feeding cholesterol without thiouracil for a 16-month period, when an average blood cholesterol concentration of 429 mg % (range 212-736) was observed. Bevans, Davidson and Abell(2)

reported a rough correlation between degree of hypercholesterolemia, length of time on experiment and extent of lesions produced in thiouracil-cholesterol-fed young dogs.

Preliminary studies in this laboratory indicated that administration of I^{131} alone would maintain an average serum cholesterol concentration exceeding 430 mg % in dogs over roughly a 12-month period. Such a preparation seemed to offer some advantages, in that the animal would remain free of the uncontrolled, generalized succinoxidase depression of thiouracil and the hepatotoxic effects of a high cholesterol diet. Even if arterial lesions were not observed after 12 months at the hypercholesterolemic level achieved, it might be possible to assess efficacy of the I^{131} treatment on the basis of altered concentrations of coronary artery and aortal cholesterol, as estimated chemically.

Methods. Each of 14 adult mongrel dogs (7 males and 7 females) was given 8 mc of

* The authors gratefully acknowledge the statistical assistance of Dr. Harry Hughes, Dept. of Biometrics, School of Aviation Med., USAF, Randolph Air Force Base, Texas.

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TABLE I. Serum Concentrations of Lipid and Lipoprotein Parameters Prior to I^{131} Administration and Corresponding Average Maintenance Levels during the Subsequent Experimental Periods.

| Dog # | Exp. period (mo) | Δ wt (%) | Lipid P (mg %) | Chol. (mg %) | Lipoproteins (mg %) | | | |
|---|------------------------|--------------------|-------------------|-----------------|-----------------------------------|-----------------------------|----------------------|-----------------------|
| | | | | | High density P > 1.063 g/cc | Low density, P < 1.063 g/cc | | |
| | | | | | | S _r 0-12 | S _r 12-20 | S _r 20-400 |
| Pre-treatment | | | | | | | | |
| 7 ♂ (means & S.E.) | | | 12.8 ± 1.3 | 149 ± 14.4 | 361 ± 45.5 | 41 ± 6.5 | 0 | 4 ± 2.7 |
| 7 ♀ " | | | 15.8 ± .93 | 190 ± 8.3 | 395 ± 65.8 | 56 ± 9.1 | 0 | 10 ± 4.2 |
| 14 ♂ " | | | 14.3 ± .82 | 169 ± 9.8 | 378 ± 43.8 | 49 ± 5.7 | 0 | 7 ± 3.3 |
| Avg maintenance levels after I ¹³¹ | | | | | | | | |
| 176 ♂ | 13 | +93 | 25.0 | 586 | 503 | 357 | 260 | 317 |
| 96 ♂ | 13 | +15 | 26.2 | 559 | 589 | 305 | 174 | 374 |
| 197 ♂ | 12 | - 9 | 13.2 | 161 | 589 | 61 | 0 | 3 |
| 635 ♂ | 11 | +38 | 23.1 | 467 | 680 | 385 | 182 | 215 |
| 526 ♂ | 11 | +21 | 19.2 | 337 | 666 | 384 | 36 | 44 |
| 580 ♂ | 11 | +16 | 18.2 | 324 | 606 | 291 | 59 | 46 |
| 220 ♂ | 11 | +71 | 24.0 | 479 | 593 | 423 | 214 | 249 |
| Avg ♂ | 11.4 | +35 | 21.3 | 416 | 604 | 315 | 134 | 178 |
| 491 ♀ | 11 | +41 | 20.6 | 410 | 595 | 424 | 44 | 52 |
| 599 ♀ | 12 | +27 | 25.7 | 562 | 634 | 487 | 171 | 91 |
| 10 ♀ | 12 | - 20 | 17.4 | 289 | 640 | 174 | 54 | 83 |
| 494 ♀ | 12 | +38 | 28.0 | 509 | 731 | 551 | 147 | 114 |
| 633 ♀ | 11 | +67 | 23.2 | 493 | 680 | 494 | 164 | 173 |
| 627 ♀ | 11 | +46 | 22.5 | 412 | 539 | 489 | 92 | 54 |
| 49 ♀ | 11 | +46 | 26.9 | 539 | 749 | 539 | 150 | 144 |
| Avg ♀ | 11.3 | +35 | 23.5 | 459 | 653 | 451 | 117 | 102 |
| Avg ♂ + | 11.4 | +35 | 22.4 | 438 | 628 | 383 | 125 | 140 |

carrier-free I^{131} in a single oral dose and maintained on commercial dog chow and water *ad libitum* over approximately a 12-month period. The animals were weighed and initial blood samples taken prior to administration of the isotope and the weighing and blood sampling were repeated at 4-week intervals throughout the year. The blood sera were analyzed chemically for levels of cholesterol and lipid phosphorus(3) and ultracentrifugally(4) for concentration of high and low density lipoproteins. The initial serum samples were labeled "pre-treatment"; the analytical results were averaged for males, females and in combination, and the means recorded in Table I. The periodic readings for each serum lipid and lipoprotein parameter recorded for each dog throughout the experimental year were combined into an "average maintenance level" (arithmetic mean of all observations) and are so listed in Table I. At term, all dogs were sacrificed by rapid ar-

terial exsanguination, autopsied and tissues were taken for analysis. The coronary artery was excised from the heart, carefully cleaned of adhering fat and connective tissue and divided at the coronary sinus. One branch was taken for histologic examination, and the other analyzed for cholesterol concentration. The aorta was similarly excised, cleaned and analyzed. A portion of the liver was taken for analysis of cholesterol concentration. 300-500 mg of tissue (wet weight) were dissolved in 1 cc of 50% aqueous KOH, the resulting colloidal sol washed quantitatively into 25 cc of alcohol-acetone and the cholesterol analysis completed in micro-glassware by the Sperry-Schoenheimer(5) technic. Repeated control studies in our laboratory demonstrated that when at least 80 γ of cholesterol were precipitated with digitonide all recoveries ranged between 95-105%. At regular intervals throughout the experimental year a total of 26 adult male dogs were sacrificed for control concen-

trations of aortal, coronary artery and liver cholesterol.

Results. Excepting dog #197 and possibly #10, the I^{131} animals developed gross signs of thyroid deficiency. The data in Table I demonstrate an average 30% increase in weight associated with a generalized edema and an average maintenance serum cholesterol concentration of 438 mg %. Correspondingly, the average maintenance levels of lipid phosphorus and all classes of ultracentrifugally determined lipoproteins are seen to be significantly higher after I^{131} administration. The alterations observed in the lipoprotein patterns are consistent with the reports of Lewis *et al.*(6). The only male-female difference in a serum parameter response was recorded in the significantly higher concentrations of the 0-12 class of lipoproteins achieved in the females. #10 was the only female for which a 0-12 lipoprotein concentration was observed at a level less than the highest value for the same serum parameter among the 7 male dogs.

Gross and microscopic examinations of the coronary arteries and aortae of I^{131} -treated dogs failed to demonstrate atherosclerotic lesions. There was no evidence of fatty degenerative or infiltrative changes in the liver. On the other hand, in the experimental male dogs, cholesterol analyses of these tissues revealed significant differences from those of the control males. Though the absolute differences are not striking, they are highly significant. The mean cholesterol concentration in the coronary arteries of the experimental male group was 2.0 ± 0.16 mg/g wet weight, as compared with the male control mean of 1.5 ± 0.076 mg/g wet weight ($P < 0.01$). Similarly, mean aortal cholesterol concentration in this experimental group was 1.8 ± 0.074 mg/g wet weight, compared with 1.5 ± 0.033 mg/g wet weight in the control group ($P < 0.01$). Since no attempt was made to separate adventitia, media, and intima and analyze these separately, it is not known if these concentration differences were uniform in the vessel wall layers, or if there was selective concentration in a portion of the vessel walls of the treated male dogs. It is possible that a selec-

tive deposition occurred in the intima, (as occurs in the natural atherosclerotic process), and that the results might have been more striking had the vessel layers been separately analyzed.

It was noted that some fatty infiltration of liver occurs after I^{131} administration that is apparently imperceptible by standard staining technics. The difference between the average of 3.2 mg/g wet weight recorded for livers of treated male dogs was significantly higher ($P < 0.01$) than the average liver cholesterol level (2.3 mg/g wet weight) found for control animals.

Comparison of the blood lipid and arterial cholesterol levels demonstrates that, in this study, there is no single blood lipid parameter that can be correlated, on an individual basis, with the levels of cholesterol found in either vessel wall at autopsy. Such is not surprising in view of the fact that only the relatively recent intimal cholesterol deposition is assumed to be the tissue parameter measured. Analysis of intimal tissue alone, or the measurement of radio-cholesterol deposition in the vessel wall would probably be more sensitive indices of recent intimal deposition. Accordingly, studies along these lines are proceeding in this laboratory.

Summary. Dogs maintained for a year after I^{131} administration were found to achieve average maintenance levels of serum cholesterol in excess of 430 mg % and comparably elevated concentrations of other serum lipid and lipoprotein parameters. Tissue analysis at autopsy revealed significantly elevated levels of coronary artery, aorta, and liver cholesterol in male treated dogs, as compared with control males. Treated female dogs differed from treated males in developing significantly higher levels of S_f 0-12 blood lipoproteins, and slightly, possibly significantly, lower levels of coronary artery cholesterol. No correlation between altered tissue cholesterol content and any of the serum lipid or lipoprotein moieties was observed.

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Behavior of Explants from Human Adult Bronchial Epithelium *in vitro*.^{*} (23645)

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During the last few years several strains of normal and carcinomatous human tissues have been maintained continuously in tissue cultures(1,2,3,4). None of these strains have been derived from non-neoplastic bronchial epithelium.

The following paper presents observations on the behavior of explants from adult human bronchial epithelium and reports the establishing of 2 permanent strains of such epithelium.

Material and methods. Samples from the bronchus of patients undergoing removal of a lung or a lobe of the lung were obtained from the operating room under aseptic conditions. The epithelium was stripped from a 1.5-2 cm length of the most proximal part of the bronchus. The small fragments, consisting of bronchial epithelium and submucosal tissue, were treated for 10-15 minutes with 5 ml of a freshly made 0.1% solution of trypsin in Hanks' salt solution containing 20 mg streptomycin and 20,000 units of penicillin. The fragments were then explanted into chicken plasma-chicken extract clot to which 40% human serum medium was added. The human serum was pooled from 2 or 3 donors, diluted with Hanks' solution containing 50 mg streptomycin and 50,000 units of penicillin per 100 ml. From the several methods of tissue-culture tried the use of micro Petri dishes(5) proved to be most satisfactory for the primary explants in our experiments. Perforated cellophane discs were put into these dishes to support the cultures since the grow-

ing cultures liquefied the clots. During 24 to 48 hours the phenol red indicator in the medium turned yellow showing acid production in the growing cultures. The medium was replaced daily or every other day by fresh 40% human serum medium. If a primary explant was established, subcultures in roller tubes were attempted after 7 to 10 days.

Results. Cultures from the bronchial epithelium of 119 adults were made. During the first 24 hours large numbers of round cells wandered to the periphery of the explant. Active movement of the cilia of ciliated epithelium could be observed. In many cultures no further migration of the cells occurred and the explant died in 4-6 days. If the explant became established, sheets of epithelial tissue grew out from the periphery of the cultures from the second day onward. The clones had blunt and rounded periphery, and the adjacent cells were without visible intercellular substance and were polygonal in shape. Many of the outgrowths ceased to increase after a few days, several survived 2 to 3 subcultures up to 40-50 days. Two permanent strains were established. Ciliation was often observed in the second or third subcultures, and both permanent strains contained ciliated epithelial cells up to the 6th generation. Such differentiation was not seen in the subsequent rapidly growing substrains. The microscopic appearance of the first outgrowth did not show any appreciable difference between the strains which ceased to grow and the 2 strains which gave rise to permanent strains.

Table I shows that apparently normal bronchial epithelium derived from persons having

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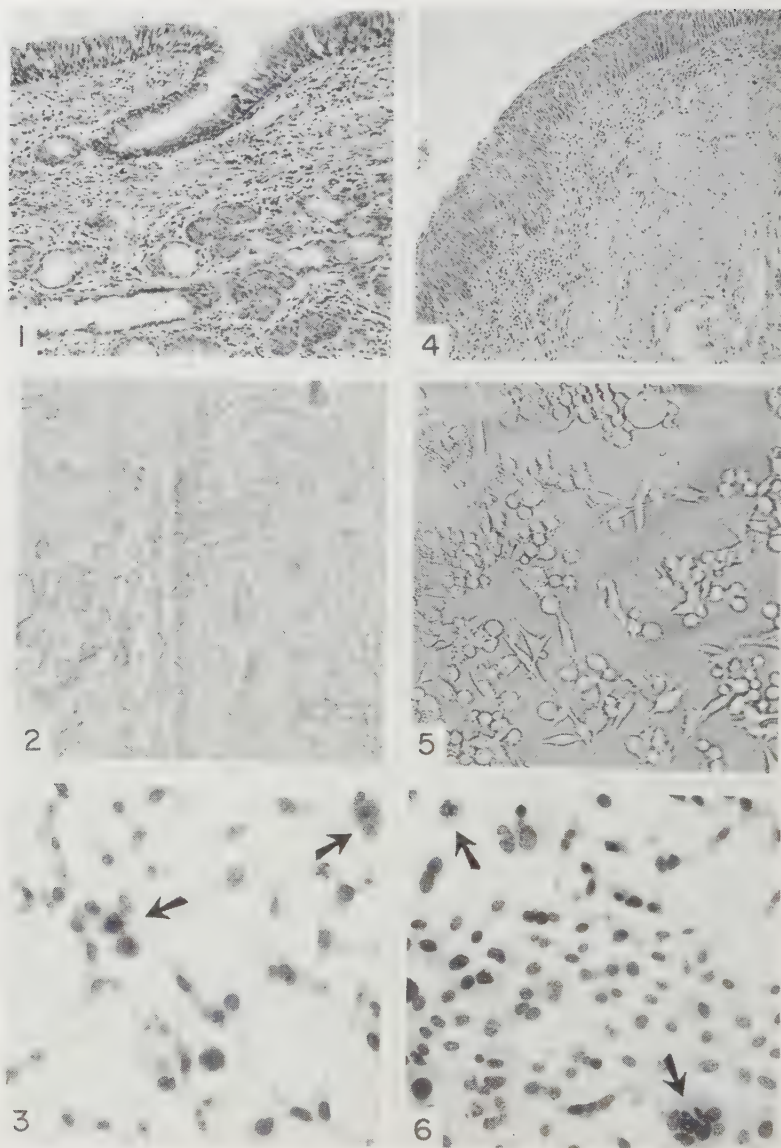


PLATE I

FIG. 1-3: Culture 130, derived from seemingly normal bronchial epithelium of a case of epidermoid carcinoma of bronchus:

FIG. 1. Segment of bronchus adjacent distally to explant; normal epithelium. Formalin-fixed, H & E, $\times 78$.

FIG. 2. Roller tube culture, 22nd generation; sheets of epithelial cells. Living, unstained, $\times 93$.

FIG. 3. "Flying" coverslip culture, 20th generation, 4 days old, showing many mitoses. Bouin's fixed, H & E, $\times 138$.

FIG. 4-6: Culture 134, derived from metaplastic bronchial epithelium of a case of epidermoid carcinoma of bronchus:

FIG. 4. Segment of bronchus adjacent distally to explant; squamous metaplasia of epithelium. Formalin-fixed, H & E, $\times 78$.

FIG. 5. Roller tube culture, 11th generation; many large cells with bizarre nuclei. Living, unstained, $\times 93$.

FIG. 6. "Flying" coverslip culture, 13th generation, 4 days old showing atypical mitosis and multinucleated giant cell. Bouin's fixed, H & E, $\times 141$.

TABLE I. Growth of Tissue Cultures of Bronchial Epithelium.

| Disease of donor | Growth of explant | | |
|---|-------------------|------|------|
| | Good | Poor | None |
| Carcinoma of bronchus | 14 | 3 | 2 |
| Pulmonary tuberculosis | 11 | 18 | 51 |
| Bronchiectasis and unresolved pneumonia | 9 | 3 | 6 |
| Blastomycosis | 1 | | 1 |

carcinoma of the lung grew generally better than analogous tissue from patients suffering from tuberculosis. Cases with unresolved pneumonia and bronchiectasis behaved in an intermediate fashion.

The segment of bronchus from which the epithelium for explant was taken was at a varying distance from the lung lesion and even in cases of bronchial carcinoma arising in a large bronchus several centimeters of grossly normal epithelium separated the tumor from the site of the explant. The microscopical examination of sections from the bronchus immediately distal from the site of the explant or of the bronchi in the whole resected lung gave no indication by which the quality of growth of the explant could be predicted. The number of epithelial layers did not differ significantly in the bronchial mucosa of tuberculous or carcinomatous patients nor in the bronchi from patients suffering from the same pathological state which did or did not give well-growing explants. In some instances, however, in the bronchial epithelium of patients with carcinoma of the lung, even in distant localization, atypical cells with large hyperchromatic nuclei and small foci of intraepithelial carcinoma were found in conformity with previous observations(6). In 2 cases of bronchiectasis the bronchial epithelium showed marked squamous metaplasia. The influence of the metaplastic or atypical changes in the epithelium on the success of the explant is difficult to evaluate since explants of 4 epidermoid carcinomas of the bronchus did grow for only a few days.

Two explants of bronchial epithelium gave rise to permanent strains. Both of these explants were derived from patients with carcinoma of the bronchus only one of whom (U. Va. 134) had demonstrable metastases.

The tissues were explanted on the 11 and 30 March, 1957 respectively.

The microscopical appearances of the cultures, U. Va. 130 and 134, are very similar to each other. The 2 cultures grew out from the explants in sheets of epithelial cells 2-3 days after explantation. A fibroblastic-like appearance of the clones was not observed. In roller tubes they formed continuous sheets of cells adherent to the wall. When growing between 2 glass surfaces in plate cultures they had amoeboid movements and diverse cell shapes could be seen. In hanging drop culture many multinuclear cells were formed. Pinocytosis and phagocytosis were observed. In appropriately stained preparations (e.g. hematoxylin and eosin, or Wright stain) many bizarre cells with hyperchromatic nuclei and anomalous chromatin arrangement were present (Plate I).

Discussion. The differences found in the explantability of bronchial epithelium of adult humans suffering from carcinoma, tuberculosis or other inflammatory diseases of the lung is thought to be connected with certain properties of the epithelium itself rather than with the treatment which the patient might have undergone prior to the removal of the bronchus. Most of the carcinomatous patients were treated with antibiotics prior to establishment of diagnosis or as a supportive measure. All of the tuberculous patients received treatment with streptomycin with or without addition of isoniazid. Since streptomycin and penicillin were added routinely to every culture without any deleterious effect, it seems unlikely that the antibiotic which the patient received should have had any influence.

Summary. Explants of bronchial epithelium were made from 119 adult human lungs removed by operation. Bronchial epithelium taken from patients with carcinoma of the lung grew more often in primary explants than analogous epithelium from tuberculous patients or from patients with bronchiectasis or unresolved pneumonia. Two explants, both derived from cases of carcinoma of the lung, gave rise to permanent strains. The histological appearance of the bronchial epithelium of the donor, even in a segment immediately distal from the explant, did not give an indi-

cation as to the growth expected in the explants.

Explantation of carcinoma of the bronchus was unsuccessful in 4 cases.

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Histopathology of Hydrocephalus Resulting from a Deficiency of Vit. B₁₂* (23646)

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A high incidence of hydrocephalus and other congenital malformations occurs in infant rats from Vit. B₁₂ deficient mothers(1). The cerebral aqueduct in the hydrocephalic brain is partially or completely occluded and a unique type of tall columnar cell is absent from the roof of the third ventricle and cerebral aqueduct(2). Brains from litter mates of hydrocephalic animals have aqueducts of abnormal size and shape although their gross appearance is normal.

This paper is an extension of previous studies made in our laboratories on the hydrocephalic brain in newborn rats from Vit. B₁₂ deficient dams. The significant observations were that the ependymal lining and choroid plexus were deranged and contained excess lipide.

Methods. Weanling female rats of the Wistar strain were fed a Vit. B₁₂ deficient diet, composed chiefly of soybean meal and glucose(1) until they reached a weight of 160-170 g. During the reproductive phase some of the animals were continued on this diet while control animals received the same

diet supplemented with 30 µg of Vit. B₁₂/kg. Thirty-two Vit. B₁₂ deficient females bore 896 offspring comprising 132 litters. Of this number 92, or about 10%, were hydrocephalic. This abnormality was detected at birth by sectioning the gross brain with a knife blade after fixation in Bouin's solution. In the normal brain there is no grossly detectable distension of the lateral ventricles. Ventricular distension in hydrocephalic brains varied from 1 to 2 mm in mild cases to 6 to 8 mm in severe cases. A higher percentage of mild cases was detected by this method than by the light transmission technic(3) and resulted in approximately doubling of the observed incidence. No hydrocephalus was demonstrated at birth among offspring of control dams. For routine observation 7 µ sections were cut and stained with hematoxylin and eosin. Brains used for fat studies were stained with osmic acid(4) and Herxheimer's fat stains(5). Brains from 34 hydrocephalic offspring, 35 litter mates and 28 controls were examined.

Results. In cases of gross dilatation of the lateral ventricles the cerebral tissue was reduced to the thinness of a membrane. Microscopically, the cortical layers were disrupted and in some areas of the lateral and third ventricles the thinned ependymal lining was folded outward. In other areas, notably in the third ventricle, the ependyma was absent and the brain tissue appeared areolar and

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† Public Health Service Research Fellow, Nat. Inst. of Neurological Diseases and Blindness.

showed distended spaces as though it were saturated with fluids. Detached and scattered cuboidal ependymal cells were morphologically similar to round cells. (Fig. 1, 2).

In less severe cases of hydrocephalus the columnar type ependymal cells normally found in many areas had been replaced by cuboidal type cells (Fig. 3, 4). Ependymal cells and

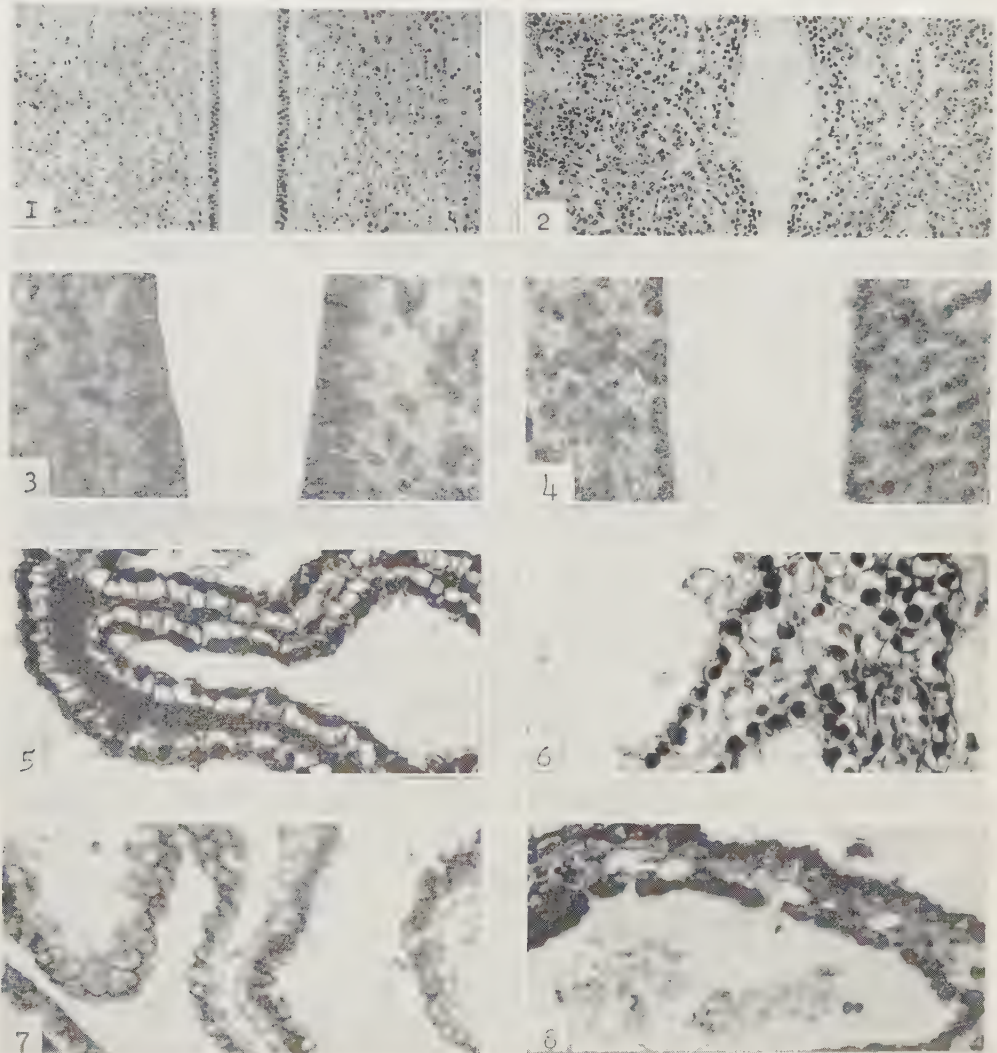


FIG. 1. Third ventricle from control rat showing normal ependymal lining. $\times 80$.

FIG. 2. Third ventricle from experimental rat with areas of ependyma entirely missing. Edema and clumping of tissue gives appearance of increase in cells. This is an artefact. The cells in tissue adjacent to the lumen are detached and scattered cuboidal type cells from the ependymal lining. $\times 80$.

FIG. 3. Third ventricle from control rat showing elongated columnar-type ependymal cell. $\times 320$.

FIG. 4. Third ventricle from experimental rat with cuboidal type cell of the thin ependyma. $\times 320$.

FIG. 5. Choroid plexus, lateral ventricle of control rat, with well defined cellular appearance. $\times 320$.

FIG. 6. Choroid plexus, lateral ventricle of experimental rat, with dilatation and fragmentation of cells.

FIG. 7. Choroid plexus, lateral ventricle of control rat. Osmic acid fat stain. $\times 320$.

FIG. 8. Choroid plexus, lateral ventricle of experimental rat, showing dilated cells filled with fat and fat stained debris in the cavity. Osmic acid stain. $\times 320$.

surface debris stained darkly with osmic acid indicating the presence of excess lipide.

The cerebral aqueduct was occluded in 19 of the 34 hydrocephalic brains examined, but was stenotic and irregularly shaped in the other 15. Tall columnar cells normally present as an intact layer in the roof of the third ventricle and aqueduct were absent in most hydrocephalic brains although they were observed in some cases with completely occluded aqueducts.

The choroid plexus in hydrocephalic brains had lost its normal architecture. The cytoplasm in many cells was disrupted and granular and exhibited large vacuoles. The centrally located nuclei were pyknotic in many instances and varied in size and appearance. The cells appeared torn and fragmentation occurred into the ventricular cavity (Fig. 5, 6). Osmic acid stain showed that the cells were filled with lipide and that much of the debris was fatty in nature (Fig. 7, 8). Herxheimer's stain on frozen sections confirmed these observations.

There were mild glial changes around the lateral ventricle and less marked changes in the area of the third ventricle and aqueduct. The number of cells per unit area around the aqueduct was 15% higher in the experimental animal. The averages of 10 areas (0.74 x 0.22 mm) of the constant depth in 4 experimental and 4 control brains were 73 and 63, respectively.

Brains from litter mates of hydrocephalic animals showed no distension of ventricles, but the aqueducts were irregular in shape. The choroid plexus and ependymal cells were affected in the same manner as those of hydrocephalic animals but to a lesser degree. The tall columnar-type cells normally found in the roof of the third ventricle and aqueduct were always present in non-hydrocephalic litter mates.

Discussion. Previous observations(2) as well as those reported here show that hydrocephalus in the Vit. B₁₂ deficient newborn rat is accompanied by stenosis or complete occlusion of the cerebral aqueduct. Whether or not the latter is the cause of ventricular dilatation or is simply associated with it cannot be determined in the light of present

knowledge of origin and absorption of cerebrospinal fluid. Evidence exists to show that the cerebrospinal fluid is formed by the choroid plexus although the mechanism is not clear(6). Hassin(7) opposes the theory that the plexuses are the site of formation of cerebrospinal fluid and holds that they act as an absorbing and purifying apparatus. Hoehn(8) showed by means of artificially induced hydrocephalus that fluids in the plexus flow in both directions and that resorption of the fluid by blood vessels of the choroid plexus takes place. Granules of iron pigment are frequently found in the choroid epithelium in cases where ventricular hemorrhage has occurred. This may be regarded as evidence of absorptive activity on the part of the plexus but does not preclude concomitant secretory activity.

The presence of large quantities of fat in the ependymal lining cells and choroid plexus, as observed in this study might have a marked effect on the rate of absorption of cerebrospinal fluid. The type of lipide present could indeed affect absorption or elaboration of fluid(9). Cerebrospinal fluid pressure in hydrocephalus due to Vit. B₁₂ deficiency may rise to 350 mm of water(3). In order for pressure of this magnitude to develop there must be an active secretion and it appears that hydrocephalus observed in Vit. B₁₂ deficient rats is the result of a decreased rate of absorption of cerebrospinal fluid. This may result from a reduction of flow through the cerebral aqueduct or impaired absorption due to fat deposition in absorptive tissues.

It has been suggested(2) that the intact layer of tall columnar cells in the roof of the third ventricle and aqueduct as seen in control animals has a secretory function and helps maintain a patent aqueduct. Although absent in many hydrocephalic brains, our results show that these cells are present above some of the completely occluded aqueducts. These cells may have been non-functional but it seems unlikely that their absence is the primary defect in this type of hydrocephalus. Globus and Bergman(10) suggested that gliosis is a common cause of cerebral aqueduct stenosis in human infants. The mild glial increase observed in this study could hardly

account for complete closure of the aqueduct. As pointed out by Russell(11), gliosis is evoked in subependymal tissues by distension of the ventricles and destruction of the ependymal epithelium. This presumably arises as a reaction to pressure and tends to counteract pressure of fluid from within the ventricle. Bruemmer *et al.*(12) found a 15% increase in the number of nuclei/g of tissue in brain homogenates from Vit. B₁₂ deficient offspring. There was no difference in the amount of deoxyribonucleic acid per nucleus, but the amount of pentose-nucleic acid per cell was decreased. It was concluded that the average brain cell in the deficient animal is smaller than normal, resulting in an increased number of cells per unit volume.

Summary. Female rats deficient in Vit. B₁₂ produced offspring with a high incidence of hydrocephalus. The cerebral cortex of hydrocephalic brains was decreased in thickness and in some areas the ependyma was missing. Ependymal and choroid cellular morphology was deranged and the cells had accumulated lipide. Tall columnar-type cells normally present as an intact layer in the roof of the third ventricle and aqueduct of control rats

were occasionally found in hydrocephalic brains with occluded aqueducts.

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Nicotinic Acid—Tryptophan Metabolism in Certain Diseases.* (23647)

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Biosynthesis and metabolism of nicotinic acid has been extensively studied(1-6) in animals and in normal humans but comparatively little work has been done on this subject in persons suffering from diseases. Conflicting claims have been made regarding the principal site of synthesis of nicotinic acid. Liver(7-11), intestinal bacteria(12-16), and body tissues(17-21) have been suggested as the major site of this vitamin formation. The present communication deals with studies on

the urinary excretions of nicotinic acid and amide, quinolinic acid, N'-methylnicotinamide (N'MN), 6-pyridone, tryptophan, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid in patients suffering from typhoid fever, cholera, small pox, liver cirrhosis, and infective hepatitis before and after feeding of tryptophan with a view to study the metabolism of nicotinic acid and its site of synthesis from tryptophan. For the sake of comparison similar studies were made on normal men also.

Methods. Ten male patients suffering from each disease who were admitted in the N. R. Sircar Medical College Hospital, Calcutta

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were selected for the study. Only those cholera patients were taken who had just passed the anuric phase. Small pox patients were in the eruptive stage. All cirrhosis patients were complicated by some degree of ascites. Patients suffering from hepatitis had marked icteric tinge. Patients suffering from typhoid fever were not given chloromycetin. The patients were fed hospital diets which did not vary from day to day during the experimental period. The 24-hour urine was collected in a bottle containing 10 cc toluene. Urinary excretions of different metabolites were determined for 2 consecutive days. At the end of second day's collection they were fed 5 g DL-tryptophan and 24-hour urine collected daily for 3 subsequent days and analysed for constituents mentioned above. Ten normal persons were also studied similarly. However, to patients suffering from typhoid fever 5 g DL-tryptophan was fed on 2 consecutive days. Nicotinic acid and amide was estimated by the method of Banerjee *et al.* (22) as modified by Nandi & Banerjee (23). Quinolinic acid was estimated after autoclaving urine in normal sulphuric acid at 15 lb. pressure for one hour and estimating the nicotinic acid formed. Quinolinic acid values were calculated by the method of Sarett (24). N'MN was estimated by the method of Carpenter and Kodicek (25) slightly modified. 6-pyridone was estimated according to the method of Rosen *et al.* (26). Tryptophan, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid were separated by paper chromatography and the chromatograms treated with Ehrlich's reagent according to the method of Dalglish (27). The colour intensity of the spots in the chromatogram was estimated with a photoelectric densitometer to give fairly quantitative results.

The results are given in Table I.

Discussion. In the diseased conditions studied urinary excretion of tryptophan was less than normal both before and after the feeding of tryptophan. This might be due to diminished absorption, increased incorporation into the body tissues, increased conversion into nicotinic acid, or increased oxidation in the tissues.

Cholera: The absence of kynurenin but appearance of 3-hydroxyanthranilic acid fol-

lowing tryptophan feeding suggests that kynurenin is metabolised to 3-hydroxyanthranilic acid at a very rapid rate. The synthesis of nicotinic acid proceeds at a subnormal rate. This might be due to deficient absorption of tryptophan from the intestine, or deficient synthesis of nicotinic acid by the intestinal bacteria which have been washed away by purging or deficient synthesis of nicotinic acid from tryptophan in tissues.

Small pox: Urinary excretion of different metabolites of nicotinic acid was very high both before and after administration of tryptophan. Possibly the breakdown of body tissues due to toxæmia makes available additional sources of tryptophan for synthesis of nicotinic acid. Higher quantities of N'MN and 6-pyridone excretions indicate an attempt on the part of body to get rid of extra load of niacin in the process of detoxication. **Liver cirrhosis:** The absence of 3-hydroxyanthranilic acid but increase in urinary excretion of nicotinic acid and its derivatives after tryptophan feeding suggests that the compound is too rapidly transformed into nicotinic acid derivatives. The increase in the values of nicotinic acid and its derivatives after tryptophan feeding indicates that synthesis of nicotinic acid is not disturbed in this disorder. Gabuzda *et al.* (28) have reported urinary excretion of tryptophan to be slightly higher than normal in this condition; our findings, however, do not agree with them. **Infective hepatitis:** Synthesis of nicotinic acid proceeds almost normally in this disease. **Typhoid:** Synthesis of nicotinic acid and its final disposal proceeds normally though at a slightly accelerated rate.

Since synthesis of nicotinic acid follows almost the normal pattern in liver cirrhosis and hepatitis, it is unlikely that liver is the organ mainly concerned with this function. This agrees with the observation of Banerjee and Basak (29) who reported that liver poisoned with carbon tetrachloride injection in rhesus monkeys did not result in reduction of nicotinic acid synthesis. Diminished synthesis and metabolism of nicotinic acid in cholera suggests many possibilities already mentioned. In typhoid where the intestine is the chief site of lesion, the synthesis of nicotinic acid

TABLE I. Average 24-Hour Urinary Excretions of Metabolites of Nicotinic Acid and Tryptophan in mg by Each 10 Patients Suffering from Different Diseases.

| | | Nicotinic acid and amide | Quinolinic acid | N'MN | 6-pyridone | Tryptophan | Kynurenin | 3-hydroxyan- thranilic acid |
|--------------------|---------------------------|-----------------------------|--------------------|------------------|------------------|-------------------|------------------|--------------------------------|
| Cholera | Before feeding tryptophan | 1.5 \pm .23* | 2.3 \pm .56 | 2.3 \pm .22 | 2.38 \pm .53 | 17.5 \pm 2.5 | 0 | 0 |
| | Days after feeding | | | | | | | |
| | 5 g DL-tryptophan | | | | | | | |
| | 1 | 2.57 \pm .34 | 5.04 \pm .48 | 4.21 \pm .31 | 13.6 \pm 2.3 | 161.5 \pm 46.1 | 0 | 34.0 \pm 4.5 |
| | 2 | 2.01 \pm .24 | 3.90 \pm .41 | 3.71 \pm .22 | 12.2 \pm 1.5 | 60.7 \pm 3.9 | 0 | 16.1 \pm 1.4 |
| | 3 | 1.68 \pm .19 | 3.02 \pm .45 | 2.85 \pm .27 | 6.4 \pm 1.0 | 22.0 \pm 2.4 | 0 | 0 |
| Small pox | Before | 11.3 \pm .94 | 12.5 \pm 1.0 | 15.7 \pm 1.2 | 9.0 \pm .7 | 0 | 0 | 0 |
| | After | | | | | | | |
| | | | | | | | | |
| | 1 | 20.8 \pm .75 | 27.5 \pm 1.4 | 32.9 \pm 1.5 | 45.5 \pm 2.6 | 165.6 \pm 3.5 | 100.8 \pm 4.0 | 40.6 \pm 2.5 |
| | 2 | 14.6 \pm 1.1 | 17.5 \pm 1.6 | 28.6 \pm 1.8 | 35.9 \pm 1.3 | 55.0 \pm 2.1 | 0 | 0 |
| | 3 | 11.7 \pm .9 | 13.4 \pm 1.2 | 21.8 \pm 1.3 | 20.4 \pm 1.5 | 13.2 \pm 1.0 | 0 | 0 |
| Cirrhosis of liver | Before | 2.51 \pm .09 | 2.44 \pm .39 | 4.35 \pm .4 | 2.46 \pm .56 | 37.1 \pm 2.6 | 0 | 0 |
| | After | | | | | | | |
| | | | | | | | | |
| | 1 | 5.08 \pm .47 | 6.14 \pm .60 | 9.15 \pm .48 | 16.6 \pm 2.0 | 723.1 \pm 32.6 | 53.8 \pm 3.2 | 0 |
| | 2 | 4.08 \pm .43 | 4.85 \pm .47 | 7.37 \pm .62 | 14.6 \pm 1.6 | 71.0 \pm 4.5 | 0 | 0 |
| | 3 | 2.91 \pm .14 | 3.18 \pm .42 | 5.22 \pm .56 | 6.86 \pm .92 | 49.6 \pm 2.8 | 0 | 0 |
| Hepatitis | Before | 3.43 \pm .36 | 2.92 \pm .24 | 7.21 \pm .48 | 1.88 \pm .3 | 0 | 0 | 0 |
| | After | | | | | | | |
| | | | | | | | | |
| | 1 | 6.98 \pm .59 | 9.51 \pm .62 | 12.8 \pm 1.0 | 17.7 \pm 1.4 | 459.0 \pm 30.6 | 52.4 \pm 3.7 | 67.4 \pm 3.5 |
| | 2 | 5.38 \pm .37 | 6.32 \pm .42 | 9.78 \pm .48 | 12.8 \pm 1.3 | 58.2 \pm 4.4 | 0 | 0 |
| | 3 | 3.9 \pm .41 | 3.58 \pm .35 | 7.69 \pm .23 | 6.0 \pm .91 | 14.2 \pm 1.1 | 0 | 0 |
| Typhoid fever | Before | 5.23 \pm .49 | 8.29 \pm 1.3 | 11.89 \pm .87 | 4.03 \pm .84 | | | |
| | After | | | | | | | |
| | | | | | | | | |
| | 1 | 10.19 \pm .63 | 14.24 \pm .80 | 19.27 \pm 1.32 | 37.61 \pm 4.49 | | | |
| | 2 | 12.47 \pm .57 | 17.86 \pm 1.1 | 26.54 \pm 1.82 | 53.68 \pm 5.81 | | | |
| Normal | Before | 4.53 \pm .21 | 3.76 \pm .50 | 7.1 \pm .41 | 3.3 \pm .82 | 63.2 \pm 2.6 | 0 | 0 |
| | After | | | | | | | |
| | | | | | | | | |
| | 1 | 7.84 \pm .29 | 10.39 \pm .59 | 13.9 \pm .78 | 45.3 \pm 6.7 | 1007.0 \pm 36.2 | 270.5 \pm 25.8 | 171.6 \pm 11.4 |
| | 2 | 6.16 \pm .31 | 4.76 \pm .38 | 10.3 \pm .56 | 30.2 \pm 4.4 | 233.4 \pm 18.2 | 0 | 0 |
| | 3 | 5.03 \pm .27 | 4.08 \pm .22 | 7.6 \pm .47 | 18.9 \pm 2.5 | 121.5 \pm 9.4 | 0 | 0 |

* Mean \pm S.E. Anthranilic acid was absent from urine in all subjects.

proceeds quite normally, which contradicts the belief that nicotinic acid is mainly formed in the gut. The enhanced rate of conversion of tryptophan into nicotinic acid and its final disposal in small pox indicates that body tissues are more concerned in the synthesis of this vitamin.

Summary. 1. Urinary excretions of nicotinic acid and amide, quinilinic acid, N'-methylnicotinamide, 6-pyridone, tryptophan, kynurenin, anthranilic acid and 3-hydroxyanthranilic acid were estimated in normal men and in patients suffering from typhoid fever, cholera, small pox, cirrhosis of liver and infective hepatitis both before and for 3 days after the feeding of tryptophan. 2. Excretion of tryptophan both before and after feeding diminished in all the diseases studied. Kynurenin which was absent initially in the urine of patients appeared in urine collected for 24 hours after the feeding of tryptophan in small pox, liver cirrhosis and hepatitis but not in cholera. 3-hydroxyanthranilic acid appeared in urine after tryptophan was fed in cholera, small pox and hepatitis but not in cirrhosis of liver. In all diseased conditions studied, except in typhoid and small pox, urinary excretion of nicotinic acid and its metabolites was greatly diminished. After administration of tryptophan urinary excretion of nicotinic acid metabolites increased in all diseases but the increase was least in patients suffering from cholera. 3. Patients suffering from small pox excreted increased amounts of nicotinic acid and its derivatives both before and after tryptophan feeding as compared to normal men.

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Corticotrophic Activity of Human Plasma Constituents.* (23648)

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The nature of corticotrophin (ACTH) as it occurs in the pituitary gland has been well studied by a variety of technics but little has been reported on the properties of this hormone as it occurs in the peripheral circulation of man, although its presence in minute amounts has been indicated(1). In this report the activity of fractions of human plasma prepared according to the method of Cohn and his associates(2,3) has been assayed for ACTH content by the bioassay technics of Nelson and Hume(4).

Methods and material. Human blood used in this study was collected into acid-citrate-dextrose (ACD) solution or through a cationic exchange resin column (Dowex 50). The plasma was separated from the red cells by centrifugation at 4000 rpm for 30 minutes at 2°C. Plasma pools from 5 to 10 donors were then subjected to fractionation with the cold ethanol methods 6 and 9(2,3). The following fractions with their major constituents as noted below were obtained: Fraction I—fibrinogen. Fraction II + III— γ -globulins and β -lipoproteins. Subfractions of II + III: III-0: β -lipoproteins: II + IIIw: $-\gamma$ -globulins, prothrombin, plasminogen. Fraction IV-1: α -globulins, cholesterol. Fraction IV-4: α and β -globulins and β_1 -metal binding globulins. Fraction V: albumin. The separated plasma fractions were dissolved in a minimum volume of 0.15 M sodium chloride and dialysed for 12 hours against 0.15 M sodium chloride at 2°C at pH 6.8 to 7.0. The samples were frozen until bioassayed. The bioassay was performed in hypophysectomized, anesthetized dogs in whom a catheter had been placed in the right lumbo-adrenal

vein in order to make possible the timed, intermittent collection of adrenal venous blood (4). ACTH activity in plasma fractions administered intravenously during 2 minutes to these animals was compared with standard ACTH given in a similar manner. One minute following the injection of the ACTH or the unknown sample, the collection of adrenal venous blood was begun and was continued for exactly 10 minutes. The response of each intravenously administered fraction was evaluated in terms of the increase in 17-hydroxycorticosteroid output by the dog adrenal over the control secretion when only the diluent saline was given. The potency and approximate 95% confidence limits of each fraction in which activity was found were calculated by the usual formulas(5) from the log-dose response curve of the increased output of steroids produced by the fraction sample and that produced by 1, 2, 5 and 8 milliunit doses of U.S.P. Standard ACTH. Because of the small quantities of ACTH present, it was not possible to construct a log-dose curve for each sample given, but values were obtained by comparison with the standard ACTH log-dose curve. Standards and unknowns were given to the same assay animal. This value has been expressed in the table as milliunits of ACTH/100 ml of reconstituted plasma; the equivalent volume of fractionated plasma injected has also been given.

Results. Studies have been performed on 3 types of human plasma: 1. plasma collected from normal human donors; 2. plasma collected from normal human donors to which U.S.P. ACTH was added *in vitro* one week following collection; 3. plasma obtained from patients and one normal donor following the intravenous infusion of ACTH (Upjohn).

Summary of the findings on 4 plasma pools collected from normal human donors in ACD or through resin is shown in Table I. ACTH activity was consistently found in Fraction

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TABLE I. ACTH Activity in Plasma Fractions from Pooled Blood of Normal Subjects.

| Plasma fraction | Pool sample No. | ACTH activity present | Adrenal venous 17-OHCS, μg per 10 min. | | ACTH, $\mu\text{u}/100$ ml whole plasma | 95% confidence limits | Fraction Equiva- lent in whole plasma | |
|-----------------------|-----------------|-----------------------|---|------|---|-----------------------|--|-----|
| | | | Control mean* | Exp. | | | Vol inj. (ml) | |
| Precipitate I | 3 | 0 | 22 | 16 | 0 | | 16 | 150 |
| | 3 | 0 | 2 | 5 | 0 | | 16 | 150 |
| " II + III | 1 | + | 11 | 52 | .3 | .06-1.9 | 20 | 300 |
| | 2 | + | 12 | 53 | .7 | .2 -2.6 | 25 | 160 |
| | 2 | + | 12 | 33 | .9 | .2 -3.6 | 12 | 100 |
| | 3 | + | 12 | 33 | .3 | .1 - .4 | 30 | 150 |
| Subfraction II + IIIw | 3 | + | 6 | 45 | .9 | .6 -1.3 | 33 | 150 |
| " III-0 | 3 | + | 6 | 59 | 1.4 | .8 -2.4 | 20 | 150 |
| Precipitate IV-1 | 1 | 0 | 4 | 4 | 0 | | 13 | 250 |
| | 1 | 0 | 2 | 4 | 0 | | 13 | 125 |
| | 2 | + | 12 | 35 | .6 | .1 -2.5 | 20 | 150 |
| | 3 | 0 | 12 | 12 | 0 | | 16 | 150 |
| | 4 | + | 1 | 25 | .3 | .08-1.0 | 10 | 150 |
| " IV-4 | 3 | + | 6 | 33 | .6 | .4 - .9 | 19 | 150 |
| | 4 | 0 | 7 | 4 | 0 | | 13 | 150 |
| " V | 1 | 0 | 9 | 18 | 0 | | 20 | 190 |
| | 1 | 0 | 2 | 4 | 0 | | 20 | 190 |
| | 2 | 0 | 17 | 17 | 0 | | 20 | 200 |
| | 3 | 0 | 7 | 11 | 0 | | 18 | 150 |

Plasma pools 1, 2 and 4 were from whole blood collected from 5-10 donors in ACD.

Plasma pool 3 was from whole blood collected from 5 donors through cationic exchange resins (Dowex-50).

* Mean of 2 or 3 collection periods following infusion of saline control.

II + III and occasionally in Fraction IV-1 or Fraction IV-4. Activity was present in both subfractions of II + III.

In the second group of experiments, 10 milliunits of ACTH were added to week-old plasma 12 hours before fractionation. Assay as shown in Table II revealed the presence of ACTH activity in Fraction II + III and also in Fraction I. The occasional contamination of Fraction I with whole plasma during the procedure could explain this result.

The assay of fractions obtained following the intravenous infusion of ACTH into 4 groups of patients gave results similar to those obtained in normal plasma except that the level of activity was higher (Table III). The plasma volume assayed from pool 2 was not large enough to give a significant increase in 17-hydroxycorticosteroid output as whole plasma or as fraction II + III. However, the larger volume given as subfractions II + IIIw and III-0 did have slight activity. In the ta-

TABLE II. ACTH Activity in Plasma Fractions following *In Vitro* Addition of 10 Milliunits of ACTH to 200 ml Pooled Plasma.†

| Plasma fraction | ACTH activity present | Adrenal venous 17-OHCS, μg per 10 min. | | ACTH, $\mu\text{u}/100$ ml whole plasma | 95% confidence limits | Fraction Equiva- lent in whole plasma | |
|-----------------|-----------------------|---|------|---|-----------------------|--|-----|
| | | Control mean* | Exp. | | | Vol inj. (ml) | |
| Precipitate I | + | 1 | 32 | 1.0 | .4-2.6 | 13 | 100 |
| " II + III | + | 1 | 21 | .8 | .3-2.05 | 25 | 100 |
| " IV-1 | — | 1 | 2 | 0 | | 20 | 100 |
| " IV-4 | — | 1 | 5 | 0 | | 20 | 100 |
| " V | — | 1 | 3 | 0 | | 25 | 100 |

* Mean of 2 collection periods following infusion of saline control.

† U.S.P. standard. ACTH added to plasma 12 hr prior to fractionation.

TABLE III. ACTH Activity in Plasma Fractions from Pooled Blood of Subjects Infused with ACTH.

| Plasma fraction | Pool sample No. | ACTH activity present | Adrenal venous 17-OHCS, μg per 10 min. | | ACTH, $\mu\text{u}/100$ ml whole plasma | 95% confidence limits | Fraction Equivalent in whole plasma | |
|-----------------------|-----------------|-----------------------|---|------|---|-----------------------|-------------------------------------|-----|
| | | | Control mean* | Exp. | | | Vol inj. (ml) | |
| Plasma control | 1 | + | 3 | 143 | 48 | 3.7 - 630 | 4 | 4 |
| | 2 | 0 | 6 | 10 | 0 | | 5 | 5 |
| | 2 | 0 | 5 | 17 | 0 | | 5 | 5 |
| | 3 | + | 4 | 41 | 18 | 7.8 - 41.05 | 7 | 7 |
| | 4 | 0 | 6 | 5 | 0 | | 20 | 20 |
| Precipitate I | 4 | 0 | 6 | 4 | 0 | | 7.5 | 100 |
| " II + III | 1 | + | 3 | 230 | 90 | 10.5 - 780 | 5 | 10 |
| | 2 | 0 | 6 | 7 | 0 | | 2 | 10 |
| | 2 | 0 | 6 | 12 | 0 | | 5 | 20 |
| | 3 | 0 | 4 | 11 | 0 | | 8 | 16 |
| | 4 | \pm | 6 | 13 | 1.0 | .3 - 3.0 | 32 | 100 |
| | 4 | + | 7 | 29 | 1.1 | .6 - 2.0 | 33 | 100 |
| Subfraction II + IIIw | 1 | + | 1 | 54 | 41 | 7.4 - 231 | 10 | 20 |
| | 2 | 0 | 5 | 4 | 0 | | 5 | 20 |
| | 2 | + | 6 | 54 | 8.6 | 2.4 - 31 | 13.5 | 18 |
| | 3 | 0 | 4 | 4 | 0 | | 9 | 27 |
| " III-0 | 1 | \pm | 1 | 16 | 8.1 | .8 - 79 | 11 | 20 |
| | 2 | 0 | 5 | 8 | 0 | | 5 | 20 |
| | 2 | \pm | 6 | 16 | 3.3 | .8 - 14 | 17 | 19 |
| | 3 | + | 4 | 78 | 4.7 | 2.04 - 10.8 | 18 | 40 |
| Precipitate IV-1 | 4 | 0 | 6 | 1 | 0 | | 25 | 100 |
| " IV-4 | 4 | 0 | 6 | 13 | 0 | | 23 | 100 |
| " V | 4 | 0 | 6 | 1 | 0 | | 23 | 100 |

Plasma pool 1 collected from a patient at the end of an 8 hr infusion of 25 units ACTH.

Plasma pools 2 and 3 collected at the end of an 8 hr infusion of 25 units ACTH, 2 patients contributing to each pool.

Plasma pool 4 collected from a normal subject 15 min. following intrav. inj. of 25 units ACTH.

* Mean of 2 or 3 collection periods following infusion of saline control.

bles, the volume actually injected into the assay animal does not necessarily correlate with the equivalent of whole plasma from which it was obtained. This is due to some technical variations in the final concentration of the particular plasma fraction which had been obtained for assay.

Discussion. ACTH activity has been shown to occur in the γ -globulin and beta-lipoprotein fractions of normal human plasma (Fraction II + III). The activity found is low and is in approximate agreement with the amounts estimated to be present in normal plasma(1).

Fraction II + III is composed primarily of protein molecules of low solubility such as γ -globulins and β -lipoproteins. Since ACTH as isolated from the pituitary gland has been shown to be a small polypeptide of high solubility(6) there is the suggestion that ACTH as found in human plasma has differ-

ent physical characteristics. If it exists as a polypeptide similar to that isolated from the pituitary, it most likely is present in plasma as a complex with a protein of a higher molecular weight. The occasional occurrence of activity in Fractions IV-1 or IV-4 may also indicate the existence of more than one ACTH complex with different solubility characteristics.

Studies in which commercial ACTH was added to human plasma *in vivo* and *in vitro* confirm the occurrence of activity primarily in Fraction II + III and suggest further the existence of ACTH in plasma as a protein-polypeptide complex.

It is of interest to note that ACTH activity has been found in the same fraction in which antidiuretic hormone, pituitary gonadotrophins(7) and insulin-like activity(8) have been found.

Summary. Fractions of human plasma prepared by methods 6 and 9 of Cohn and his associates have been assayed for ACTH activity by the procedure of Nelson and Hume. ACTH activity was found in Fraction II + III and occasionally in Fraction IV-1 and Fraction IV-4. Fractionation of human plasma to which ACTH had been added *in vivo* and *in vitro* gave similar results.

The authors are indebted to Mrs. Nancy Meegan, Misses Anita Eisold and Norma Madiera for technical assistance.

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Rapid Slide Precipitin Microreaction of Poliomyelitis Antigens and Antisera in Agar.* (23649)

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(Introduced by A. B. Sabin)

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The precipitation of viral suspensions by specific antisera has been applied to plant viruses, and recently also to animal viruses. Belyavin(1) described the precipitation of influenza virus in aqueous suspension by means of specific antisera. Wilson Smith *et al.*(2) reported a flocculation reaction with polioviruses. Since their original method required large amounts of antigen, the same authors developed a micromethod of immuno-precipitation of polioviruses in aqueous medium in capillary tubes(3). Le Bouvier(4), as well as the present authors(5) independently described the use of the Ouchterlony method of double diffusion in an agar gel(6) for the study of the reaction between polioviruses and specific antisera. Since the usual technic in Petri dishes requires large quantities of antigen, a micromethod, described elsewhere(7) for the study of venoms and other antigens,

was applied to the polioviruses.

Materials and methods. Routine microscopic slides, cleaned in alcohol, are used. Each slide is covered with 3 ml of a 1% hot agar solution. After solidification, 2 mm holes are punched in the agar with a special instrument. A fixed pattern of 6 holes disposed around a seventh, as shown in Fig. 1, proved to be very useful. The volume of each hole is approximately 4 cu. mm. **Agar solution.** A 2% solution of commercial, pure agar in distilled water is clarified by precipitation with egg albumin at 120°C. For use, this agar solution is melted and diluted to 1% in a M/7.5 phosphate buffer solution (Sørensen) and while very hot poured on the slides. The final pH should be 7.2. This concentration of agar was chosen in accord with Polson's data(8), because it is the minimal amount of agar giving a sufficiently solid sheet. **Antigens.** Cultures of KB cells in large flasks are massively infected with the respective type strains in use here (type 1 = strain No. 6,186 from Dr. Goffe; type 2 = strain MEF₁; type 3 = strain Saukett). In

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a medium consisting of 0.5% lactalbumin hydrolysate in Earle's solution, supplemented with 10% calf serum, the degeneration of the cells is almost complete in 24 hours. The flasks are then frozen and thawed, and the contents are clarified by 2 centrifugations at 3,200 rpm for 20 minutes. The viruses are then concentrated by centrifugation on the Spinco preparative machine at 30,000 rpm for 90 minutes using lusteroid tubes in the 40 rotor. The supernatant fluid is discarded, the pellets are suspended in PBS buffer(9) and left at 4°C overnight. Repeated pipetting then yields a uniform suspension. The tubes are rinsed with PBS and the pooled suspensions are adjusted to give a 50-fold concentration. This antigenic suspension is opalescent and contains no gross particles. Titration of the original suspension should give titers of at least 10^7 TCID₅₀ per ml. The antigens were kept at 4°C. *Sera.* As reference sera, we used the 3 type specific sera from hyperimmunized monkeys prepared by the National Foundation for Infantile Paralysis, and kindly distributed by this organization, to which we are indebted for their generous assistance. The study included also sera from patients with a clinical history of poliomyelitis, from healthy and vaccinated children and from animals (guinea pigs and rabbits) immunized with increasing volumes of Salk's vaccine in 3 courses of biweekly injections. Human gamma globulins were also used. *Procedure for reaction.* The holes in the agar are filled with the reagents by means of a doubly drawn, fine-tipped Pasteur pipette. The fundamental reaction unit consists of 2 holes containing respectively antiserum and antigen. The hexagonal array gives a constant distance between all the holes in the agar and thus gives several possibilities of reaction units. For the purpose of economy, it is advisable to use the central hole as one member of several reaction units, the other members being in each of the peripheral holes (Fig. 8). The slides are then put in a tightly closed glass chamber, containing an atmosphere that is saturated with water vapor by means of moistened blotting paper at room temperature (20°C). Tests were also made at 37°C with a slight gain in the speed of the

reaction. After 16 to 24 hours the precipitation lines which appear in the agar separating the two holes containing the specific reagents may be observed by macroscopic dark field illumination. These lines show a tendency to regress when the diffusion time is prolonged. *Drying and staining.* The slides are arranged on a glass plate and covered with a sheet of moist blotting paper, which should adhere closely to the agar surface. Care must be taken not to introduce or to leave air bubbles in the holes, because they may cause breaks in the agar sheet during desiccation. The preparations are thus kept overnight in an incubator at 37°C. They can be kept dry as long as one wants. For staining, one moistens the blotting paper with water until it can be removed. The surface of the slides is cleaned with moist cotton wool or under gentle running water. This procedure allows the agar sheet to absorb the amount of water that is needed for staining. Experience has to be gained in achieving the right degree of humidity. Slides are introduced for 2 to 3 minutes in the staining mixture, then for several seconds in a series of 4 to 5 washing baths of similar composition (the baths contain one of the solvent mixtures A or B according to the dyes used). They are kept a little longer in the last bath with manual agitation. The time for the total differentiation is only a few minutes. This procedure should produce almost complete discoloration of the agar sheet and keep the lines stained. Precise time relationships cannot be furnished, because the amount of dye in the washing baths influences the speed of washing. The last bath should be renewed frequently. *Staining and solvent mixtures.* Several dyes can be used. The following substances were found particularly suitable: *Amidoschwartz 10 B* (Bayer), 0.1% in the solvent mixture A. *Azocarmin B* (National Aniline Division), 0.5% in solvent mixture A. *Bromophenol Blue*, 1% in solvent B (after staining and differentiation, the color of the preparation is developed in ammonia vapor). *Solvent Mixture A:* Methanol 98%, 50 parts; Distilled water, 40 parts; Acetic acid, 10 parts. *Solvent B:* Ethanol 70%.

Results. a) Reference sera. When undiluted reference monotypic sera are used in

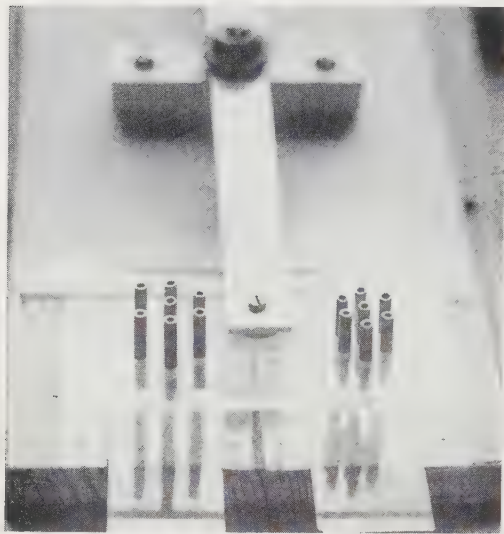


FIG. 1. Equipment used for punching holes in the agar.

this system, one observes one or two precipitation lines only with the antigen of the corresponding type. Two lines differing in intensity were observed in the type 1 (Fig. 3) and in the type 2 systems (Fig. 4), but only one in the type 3 system of precipitation (Fig. 5). The use of diluted sera shows that the precipitation lines occur at the 1/8 dilution, but are less apparent at the 1/16 dilution (Fig. 8).

b) *Gamma globulins of human origin.* With pooled gamma globulin of American or Swiss origin, precipitation lines were observed with all three types of antigen (Fig. 6). If the same solution of gamma globulin diffuses against the 3 separate types of antigen, placed in adjacent holes around the globulin, the precipitation lines cross each other. On the other hand, when several adjacent holes are filled with the same antigen, the gamma globulin gives rise to a continuous line of precipitation (Fig. 7). The main antigen or antigens of each type of poliovirus is clearly different as seen by this method.

c) *Animal sera.* The sera of rabbits and guinea pigs vaccinated with the Salk vaccine give rise to precipitation lines against the 3 monotypic antigens. No reactions were obtained with control antigens prepared from extracts of uninfected KB or monkey kidney

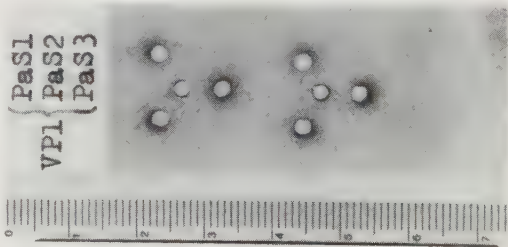


FIG. 2. Slide showing micro-reactions after staining.

TABLE I. Neutralizing and Precipitating Antibodies for Type 1 Poliovirus in Paired Sera.

| Type of individual | No. | Before vaccine | | | | | | After vaccine† | | | | | |
|--------------------|---------|----------------|----|------|----|----|---|----------------|----|------|----|----|---|
| | | NA | | PA | | | | NA | | PA | | | |
| | | 4* | 40 | Und. | 2 | 4 | 8 | 4 | 40 | Und. | 2 | 4 | 8 |
| Healthy children | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| inoculated with | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ++ | + | + | 0 |
| 3 doses of killed | 3 | 0 | 0 | + | + | 0 | 0 | 0 | 0 | + | + | + | 0 |
| poliovirus vac- | 4 | 0 | 0 | + | + | + | 0 | + | + | ++ | ++ | ++ | + |
| cine | 5 | 0 | 0 | ++ | ++ | + | 0 | + | + | ++ | ++ | ++ | + |
| | 6 | 0 | 0 | 0 | 0 | 0 | 0 | + | + | 0 | 0 | 0 | 0 |
| | 7 | 0 | 0 | 0 | 0 | 0 | 0 | + | + | + | 0 | 0 | 0 |
| | 8 | + | + | ± | 0 | 0 | 0 | + | + | ++ | ++ | + | 0 |
| | 9 | + | + | + | 0 | 0 | 0 | + | + | ++ | + | 0 | 0 |
| | 10 | + | + | ++ | + | 0 | 0 | + | + | ++ | ++ | + | 0 |
| | 11 | + | + | + | + | | | + | + | ++ | ++ | ++ | |
| | 12 | + | + | ++ | ++ | + | 0 | + | + | ++ | ++ | ++ | + |
| Serum | | | | | | | | | | | | | |
| "Non-polio" lymph- | Acute | + | 0 | 0 | 0 | 0 | 0 | | | | | | |
| | Conval. | + | 0 | 0 | 0 | 0 | 0 | | | | | | |
| Type 1 paralytic | Acute | + | + | ++ | + | + | 0 | | | | | | |
| | Conval. | + | + | ++ | ++ | ++ | + | | | | | | |

NA = neutralizing antibody; PA = precipitating antibody. In precipitation tests, + = definite reaction and ++ = heavy precipitation.

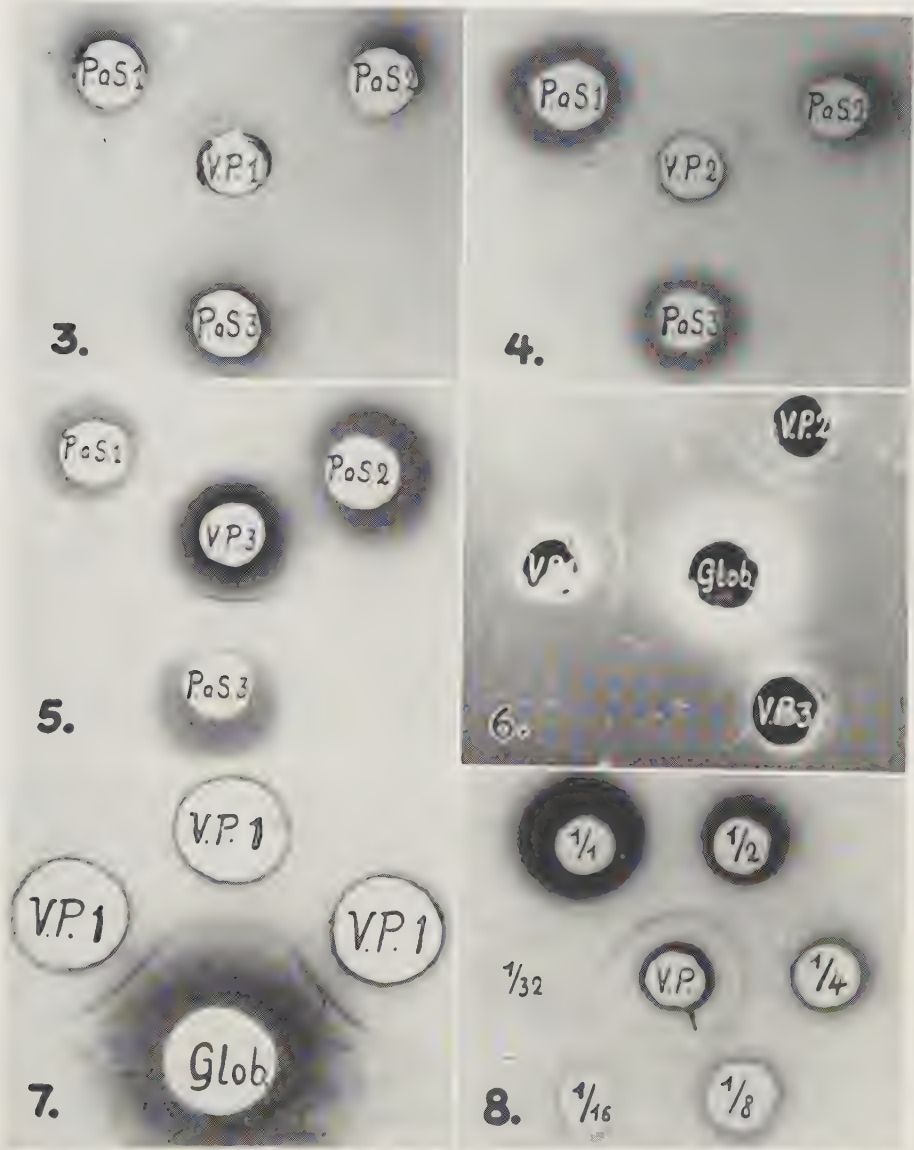
* Dilution of serum tested. † 4 wk after 3rd dose.

cells. Sera from non-immunized rabbits and guinea pigs gave no reaction with poliovirus antigens.

d) *Human sera.* Twelve pairs of sera of children, taken before and after vaccination with 3 injections of a killed poliovirus vaccine were submitted to the precipitation test. One pair of sera (early and convalescent) from a child suffering from lymphocytic meningitis of

unknown, but surely not poliomyelitic origin, and one pair of sera (10- and 20-day) of a paralytic case of type 1 poliomyelitis were also tested. Results are shown in Table I.

Among the 7 children, who before vaccination had no neutralizing (N) antibody in the low dilution of serum tested, 3 (Nos. 3, 4 and 5) gave positive tests for precipitating (P) antibody. Control tests with antigens from



FIGS. 5 to 8. Enlarged photographs of different stained precipitin micro-reactions, except Fig. 6 observed unstained in darkfield. V.P. 1 = poliovirus type 1; V.P. 2 = poliovirus type 2; V.P. 3 = poliovirus type 3; P.a.S. 1 = poliomyelitis antiserum (monkey) type 1; P.a.S. 2 = poliomyelitis antiserum (monkey) type 2; P.a.S. 3 = poliomyelitis antiserum (monkey) type 3; Glob. = human gamma globulin.

uninfected KB cells were negative. Whether this is due to the presence of group-specific P antibodies in human sera for group-specific poliovirus or other viral antigens or whether in some individuals the type-specific P antibody may be a more sensitive indicator of previous infection with a specific type of poliovirus than the cytopathogenic test for N antibody used here, is not clear from the available data. The failure of child No. 3 of this latter group, who exhibited only P antibodies prior to vaccination, to develop any N antibodies after 3 doses of vaccine is not compatible with the assumption that the prevaccination P antibodies in this child were due to a previous infection with Type 1 poliovirus or for that matter with any poliovirus. Of the 7 children who exhibited no Type 1 N antibodies prior to vaccination, 3 failed to develop such antibodies after 3 doses of this particular killed virus vaccine, while one (No. 2) did develop P antibodies. Of the two children (Nos. 6 and 7) who exhibited neither N nor P antibodies prior to vaccination both developed N antibodies but only one exhibited a trace of P antibody after vaccination. There was thus no indication that the precipitin test was just another way of measuring N antibody. Among the 5 children who had Type 1 N antibody prior to vaccination all exhibited some P antibody before the vaccine and an increase in intensity of the precipitin reaction as well as a 2-fold increase in titer after the vaccine. A similar increase in the activity of the P antibody was observed in the sera from the patient with poliomyelitis, while no P antibody appeared in the sera of the one patient with lymphocytic meningitis of non-poliomyelitic etiology. Many other single serum specimens from patients with poliomyelitis were also tested and all reacted strongly in the precipitation test.

Stability of the antigen. The live concentrated virus suspensions were stored at 4°C for more than 2 months without loss of precipitating activity. In view of the potential danger of infection with these concentrated antigens, preliminary tests were carried out with formalinized antigens. Although the formalinized antigens appeared to be as po-

tent as the live virus antigens, they lost the capacity to yield the double precipitation lines observed with types 1 and 2 antisera, and the single line that occurred was more diffuse than with the live virus antigens.

Discussion. The type specificity of the precipitation lines produced in agar when poliovirus antigens were allowed to react with antibody was clearly evident when monkey antisera were used. The presence of Type 1 P antibody in the sera of 3 of 7 children who had no type 1 N antibody in the low dilution of serum tested, indicates that the question of group-specific P antigens and antibodies needs to be investigated. It is well known that group-specific, complement-fixing antibodies are readily demonstrable in human sera but not in monkey antisera containing high titers of N antibody. It is already evident that the precipitation test is not merely another way of measuring N antibody but the relationship between the P antibodies and the type-specific and group-specific complement-fixing antibodies still remains to be determined.

Summary. 1. A simple and rapid technic is described for demonstrating a precipitin reaction in agar between minute amounts of poliovirus antigens and antisera. The preparations can be dried and stained and preserved indefinitely. 2. With monkey antisera the reaction was type-specific, while human sera were encountered which exhibited the precipitating antibody in the absence of readily demonstrable neutralizing antibody for the same type of poliovirus. 3. Sera from patients with poliomyelitis and human gamma globulin regularly exhibited the precipitin reaction.

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Metabolism of Human Amnion Cell Cultures Infected with Poliomyelitis Virus. I. Glucose Metabolism During Virus Synthesis.[†] (23650)

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Human amnion cell cultures have been shown to be highly susceptible to infection with various polioviruses(1,2). Thus, a convenient system is at hand for studying metabolic changes in host cells during infection with the virus. In this work glucose uptake of infected and control cell cultures was measured, and with the aid of various metabolic inhibitors reactions important for virus reproduction were indicated.

Materials and methods. Preparation of amnion cell cultures. The technic deviates in a number of details from procedure described by Zitcer *et al.*(1), and modifications by Takemoto and Lerner(3), Weinstein *et al.*(4) and Lahelle(5). Human placentas[†] collected in sterile towels were brought to laboratory within a few hours after delivery. The amnion membrane was stripped off, washed several times with phosphate buffered saline (PBS) and blood clots were removed. The membrane was then cut into 3-4 pieces, put in Erlenmeyer flasks, and covered with pre-warmed Hanks' salt solution containing 0.25% trypsin (N.B. Co. 1/300), pH-7.6. After 1 hour, trypsin was poured off and replaced by fresh pre-warmed 0.1% trypsin solution (pH-7.6) and the amnion left overnight at room temperature. Flasks were always kept tightly stoppered. The next morn-

ing fresh trypsin solution was added and the flasks vigorously shaken by hand. This procedure was repeated 3-4 times, employing fresh (0.1%) trypsin solutions each time, till most cells were set free. The combined fluids were centrifuged at 800 rpm 8 minutes, and the sediment washed twice with sterile PBS. Total count of cells was determined in hemocytometer after staining with crystal violet solution(6), while percentage of non-viable cells was estimated from another sample stained with trypan blue(7). Suspensions showing more than 10% cells stained with trypan blue were discarded. Finally, cells were resuspended to contain 500,000/ml in a medium consisting of 4 parts of 0.5% lactalbumin hydrolysate in Hanks' solution and 1 part of calf serum. The pH of medium was adjusted to 7.4. As seed 0.5 ml of this suspension was used for tubes, and 13 ml and 50 ml for milk bottles and Roux flasks, respectively. All containers were stoppered with rubber and incubated at 37°C; tubes were kept in slightly slanted position. After 48 hours the medium was replaced by Hanks' solution containing 0.5% lactalbumin hydrolysate to which 2% calf serum and 0.5% chick embryo extract were added; 1 ml of this medium was dispensed into tubes. A confluent sheet of cells was obtained within 3 to 5 days. The maintenance medium for these cultures was composed as follows: 0.5% lactalbumin hydrolysate in Earle's salt solution and 2% of calf serum. All solutions and media contained 100 units of penicillin and 100 μ g of streptomycin/ml. Mycostatin (30 units/ml) was added to membranes during

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[†] Obtained through the courtesy of gynecological staff of General Bikur-Cholim and Misgav Ladagh hospitals, Jerusalem.

[‡] Aided by a grant from the estate of Miss Lutie D. Goldstein, San Francisco.

overnight trypsinization and to all media. After infection of cell cultures with poliovirus, a medium was added which contained amino acids and vitamins in concentrations as used by Eagle for cultivation of HeLa cells(8), Hanks' solution and glucose 500 $\mu\text{g}/\text{ml}$. However, it contained no serum. *Monkey kidney cell cultures.*[†] Some experiments were performed with rhesus monkey kidney cells in addition to those using amnion cell cultures. For this purpose one Roux bottle containing a monolayer of trypsinized monkey kidney cells was used. The cells were harvested by the versene method(9), and grown in test tubes 3-4 days in medium 199, till monolayers were formed. *Virus.* The MEF₁ strain of poliovirus type II was used. Pools of the virus were prepared from infected amnion cell cultures grown in Roux bottles. The supernatant containing the virus was separated from cell debris by centrifugation, and its titer determined by 10-fold dilutions in test-tube-cultures by examination of the cytopathogenic effect. Two to 4 tubes were used/dilution, and the TCID₅₀ was determined by Reed & Muench method(10). A titer of $10^{7.0}/\text{ml}$ was usually obtained. The virus was preserved at -20°C . In a number of experiments inactivated virus was employed. For this purpose virus suspensions were heated in water bath for 30 minutes at 56°C , or for 5 minutes at 100°C . At both temperatures complete inactivation of virus was obtained. Neutralization of virus with immune monkey serum[‡] was also performed. Equal volumes of virus suspension and serum diluted 1:5 were mixed and kept at room temperature for 30 minutes. A control of normal monkey serum was also employed. No rise of virus titer was observed following infection of cultures with immune serum treated virus, whereas normal serum had no inhibiting effect on virus production. *Infection of cultures.* Pooled fluids of 10 test tube cultures or of one milk bottle were examined. Repeated counts gave values of 2×10^5 cells/

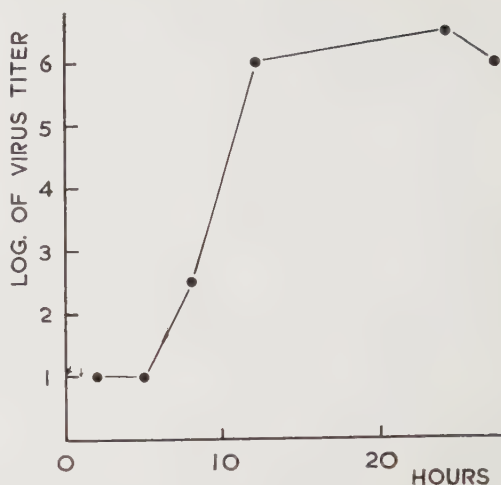


FIG. 1. Growth curve of MEF₁ strain of poliovirus type II in human amnion cell cultures. Cell cultures incubated with poliovirus for 1 hr at 37°C , then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

tube, and 2-3 $\times 10^6/\text{milk bottle}$. Infection of cultures was carried out by adding sufficient virus to give a multiplicity of 5. The virus was left in contact with cell cultures for 1 hour at 37°C . The cultures were then washed 2-3 times with PBS to remove unadsorbed virus, the modified medium of Eagle was added and cultures reincubated at 37°C . Aliquots were taken immediately after addition of new medium and after various time intervals (1, 3, 5, 7, 9, and 24 hours). Uninfected control cultures were similarly treated. Appearance of cytopathogenic changes due to virus multiplication, was followed by microscopic examination. At the same time determinations for presence of glucose and phosphorus in the aliquots were also made. Glucose was examined by the Somogyi-Nelson photometric method(11) and phosphorus by the Fiske-Subbarow method(12). *Inhibitors.* The influence of a number of inhibitors on glucose uptake by control and infected cell cultures was examined. Potassium cyanide (KCN), sodium azide (NaN_3), sodium fluoride (NaF), sodium monofluoroacetate (NaFAc), and monoiodoacetic acid (IAc) were used in concentration of M/100-M/1000. Solutions of inhibitors were prepared freshly in PBS. Inhibitors were added 0.1 or 0.2 ml to cell cultures immediately after addition of Eagle modified medium. Controls

[†] We are indebted to Dr. N. Goldblum, Virus Laboratory, Ministry of Health, Tel-Aviv, for monkey kidney cultures and medium 199.

[‡] Obtained through the courtesy of Dr. J. L. Melnick, Yale University, New Haven, Conn.

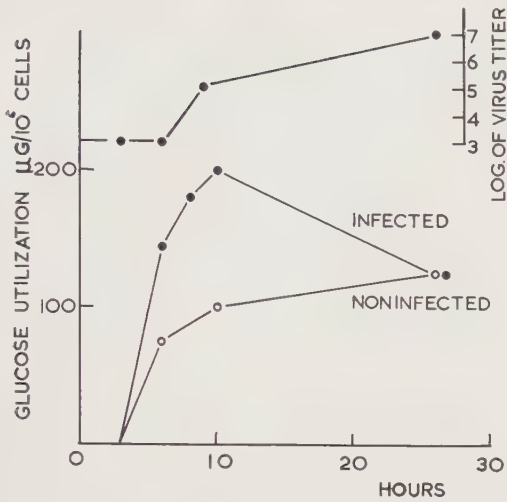


FIG. 2. Glucose uptake by non-infected and infected human amnion cell cultures during poliovirus production. Cell cultures incubated with poliovirus for 2 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

were supplemented with same amounts of PBS which were found without effect on cell metabolism; aliquots for analysis were taken at intervals.

Results. Virus multiplication. A typical growth curve of the virus is given in Fig. 1. After a lag period of 3 hours the amount of virus in the culture fluid increased steadily

TABLE I. Uptake of Glucose by Infected and Control Human Amnion Cell Cultures.

| Exp. | | Hr postreincubation* | | | | | |
|------|----------|----------------------|-----|------|------|------|------|
| | | 1 | 3 | 5 | 7 | 9 | 24 |
| 1 | Infected | 180† | 160 | 240 | 320 | 400 | 480 |
| | Control | 100 | | 120 | 260 | 320 | 400 |
| 2 | Infected | 0† | 200 | 240 | 380 | 200 | |
| | Control | 0 | 180 | 240 | 240 | 240 | |
| 3 | Infected | | 145 | 180 | 200 | | 125 |
| | Control | | 75 | 145 | 100 | | 125 |
| 4 | Infected | | 142 | 105 | 105 | | 137 |
| | Control | | 0 | 67.5 | 167 | | 228 |
| 5 | Infected | 0 | | 7.5 | 45.0 | | 77.5 |
| | Control | 0 | | 25.0 | 27.5 | | 77.5 |
| 6 | Infected | 0 | | 0 | 12.5 | 22.5 | 22.5 |
| | Control | 0 | | 0 | 0 | 12.5 | 35.0 |

* Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

† Results of cumulative glucose disappearance are expressed in $\mu\text{g}/10^6$ cells originally present in culture.

‡ 0 = no glucose consumed.

until 12 hours after infection. In other amnion cultures release of the virus was delayed for 3-7 hours, but maximal titer of virus was reached about 12 hours thereafter.

Uptake of glucose. Aliquots that were used for determinations of virus titer were also analyzed for glucose content. Results are given in Table I and a typical curve in Fig. 2.

TABLE II. Glucose Uptake by Human Amnion Cell Cultures, Non-infected and Infected with Active and with Neutralized Poliovirus.

| Hr post-reincubation* | Cell cultures | | |
|-----------------------|---------------|----------|---------------------------------|
| | Non-infected | Infected | Infected with neutralized virus |
| 1 | .0† | 10.7† | .0† |
| 3 | .0 | 23.6 | .0 |
| 5 | .0 | | .0 |
| 7 | .0 | 40.7 | .0 |
| 9 | .0 | 30.1 | .0 |
| 21 | 15.7 | .0 | 22.5 |
| 28 | 29.0 | .0 | 22.5 |

* Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

† No. represents uptake of glucose in %, cumulative from time 0, postreincubation.

Different amnion preparations showed considerable variations in glucose uptake. However, in all experiments the infected cell cultures consumed glucose more vigorously than non-infected cultures. It seems of significance to note that this increased glucose uptake precedes liberation of virus into the culture fluid, and it ceases during liberation of virus from injured cells. On the other hand, an undisturbed utilization of glucose is found in control cell cultures. In some experiments, virus release was accompanied by an increase of reducing substances in the culture fluids. No stimulation of glucose uptake was observed when cells were brought in contact with heated inactivated virus or when virus production was prevented by addition of specific antiserum (Table II).

Utilization of inorganic phosphate. As with glucose, the uptake of phosphorus was higher in infected cultures as compared to the controls. However, with liberation of free virus, a simultaneous release of inorganic

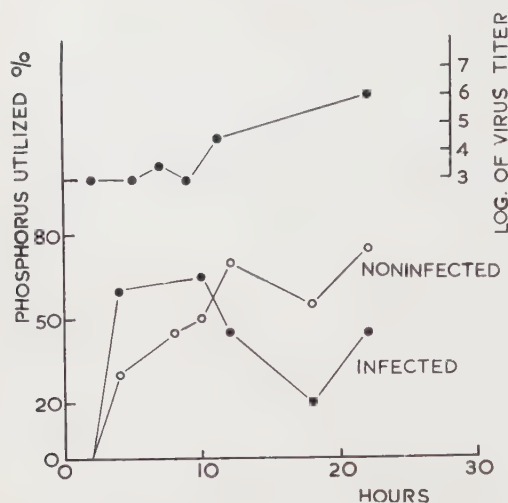


FIG. 3. Uptake of inorganic phosphorus by non-infected and infected human amnion cell cultures during virus production given as percentage of phosphorus in medium added after washing. Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

phosphate was found in the culture fluids (Fig. 3).

Effect of inhibitors on glucose metabolism and virus multiplication in amnion cell cultures. Potassium cyanide (M/1000) neither affected the viability of cell cultures nor the synthesis of poliovirus within infected cell cultures; virus yield was equal in cyanide treated and untreated cultures. Both onset and extent of the characteristic cytopathogenic effect were the same in presence or absence of

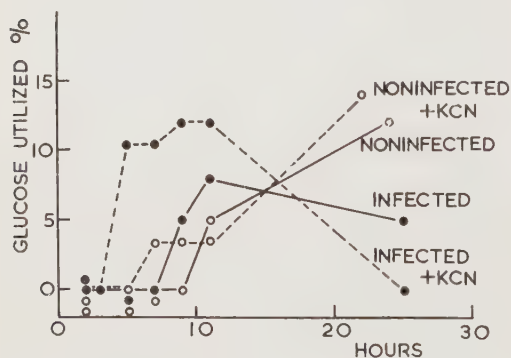


FIG. 4. Effect of cyanide (M/1000) on glucose uptake by non-infected and infected human amnion cell cultures given as percentage of glucose in medium added after washing. Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

the inhibitor. However, addition of cyanide stimulated glucose utilization of infected cultures in comparison to the controls (Fig. 4). Addition of NaN_3 (M/1000) and NaFAC (1 mg/ 10^6 cells) did not affect, within observation period of 48 hours, the viability of amnion cell cultures. In the presence of inhibitors the same cytopathogenic changes appeared as in infected non-treated cultures. As in the case of cyanide, glucose utilization was increased in infected cultures by addition of azide or NaFAC. In contrast to the effects of cyanide, azide and fluoroacetate, NaF and moniodoacetic acid (IAC) depressed glucose uptake in both control and infected cell cultures (Table III). Cultures so treated showed early cytological damage, but no cytopathogenic changes attributed to virus multiplication were observed. No multiplication of virus was found to occur in cell cultures inhibited by IAC.

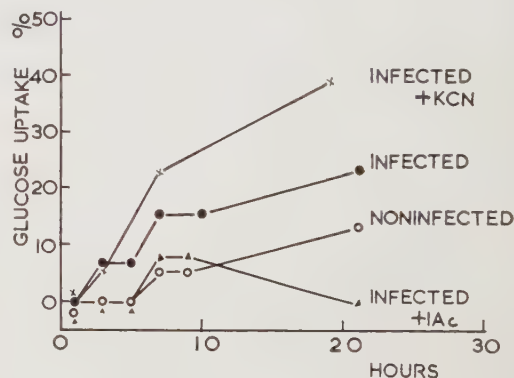


FIG. 5. Glucose uptake by non-infected and infected monkey kidney cell cultures given as percentage of glucose in medium added after washing. Effect of cyanide (M/1000) and iodoacetic acid (M/100) on glucose uptake by infected cells. Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

Glucose metabolism in infected and control monkey kidney cell cultures. The same methods as used for human amnion cell cultures were employed. Kidney cell cultures infected by poliovirus showed stimulation of glucose uptake as compared to non-infected cell cultures. The data are given in Fig. 5. As with amnion cell cultures KCN (M/1000) stimulated and IAC (M/100) inhibited glucose uptake by infected cell cultures.

TABLE III. Inhibition of Glucose Uptake by M/1000 Iodoacetic Acid and NaF in Human Amnion Cultures, Non-infected and Infected by Poliovirus.

| Hr post-reincubation* | Infected cultures | Non-infected cultures | Infected + NaF | Non-infected + NaF | Infected + IAc | Non-infected + IAc |
|-----------------------|-------------------|-----------------------|----------------|--------------------|----------------|--------------------|
| 3 | 35 † | 0 | 0 | 0 | 0 | 0 |
| 5 | " | 0 | 14.7 | 0 | 14.7 | 0 |
| 7 | " | 35 | " | 14.7 | " | 0 |
| 9 | 44.8 | " | " | " | " | 14.7 |
| 19 | 64.7 | 64.7 | 35 | 35 | " | " |

* Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus and reincubated after addition of modified Eagle medium.

† Results of cumulative glucose disappearance are expressed in $\mu\text{g}/10^6$ cells originally present in culture.

Discussion. Experiments on growth of MEF₁ poliovirus in human amnion cell cultures indicated that a latent period of 3-7 hours, during which infected cells apparently propagated virus without releasing it into the medium, followed adsorption of virus to cells. Thereafter, virus titer of the supernatant culture fluid increased rapidly to a maximum approximately 7-12 hours after the end of the latent period. Similar results have been obtained with monkey kidney and HeLa cell cultures(13,14), while Dunnebacke(15,16), working with amnion cultures, obtained a slower growth rate.

Glucose is utilized more rapidly by infected cells as compared to non-infected cultures. This is particularly evident in early hours(1-3) after infection, when the titer of the virus in the culture fluid is still low, and no cytopathogenic effect is seen (Fig. 2). With the manifestation of the cytopathogenic effect and the rise of the virus titer an increase in the reducing capacity of the culture fluid is found. This may be interpreted either as a release into the medium of cell-bound glucose from infected cells, or as a release of reducing substances other than glucose. Since glucose is an easily metabolizable substrate, the former possibility is preferred. When heat or antiserum inactivated virus was applied to cell cultures instead of active virus, glucose uptake was identical to that of the control cultures. The increased uptake of glucose may be, therefore, related to the presence of active virus production in the cells. These findings are in disagreement with those of Franklin *et al.*(17) who found a diminished uptake of glucose in poliovirus-infected

cultures. In their work, however, tissue cultures from human embryonic brain and cord were used, and glucose determinations were made after much longer intervals. Eagle and Habel(18) who determined the need of glucose and glutamine for virus synthesis by HeLa cells, postulated that the carbons of these substrates participate in the synthesis of viral nucleic acids by the host cells. The increased uptake of glucose by human amnion cell cultures upon infection with poliovirus is in agreement with this hypothesis (18).

Glucose uptake by control and infected cell cultures was stimulated by KCN, NaN_3 and NaFAC, however, infected cells utilized glucose more rapidly. This increase in glucose uptake should be attributed to increase in rate of glycolysis of the infected cells induced by blocking Krebs' cycle reactions. This interpretation is also favored by the enhanced uptake of inorganic phosphate as well as the augmented incorporation of P^{32} by HeLa cells infected with poliovirus type I(19). Since virus multiplication is not affected by inhibition of oxidative reactions, the importance of glycolysis for virus synthesis becomes even more evident.

Gifford and Syverton recently reported(20) that anaerobic conditions allow replication of poliovirus in HeLa and monkey kidney cells. Levy and Baron(21) showed increased lactic acid formation by monkey kidney cultures infected with Saukett strain of poliovirus type III. The stimulation of lactic acid formation took place under conditions of anaerobic and aerobic glycolysis. Thus, a similarity to our results obtained with human

amnion and monkey kidney cell cultures is evident. We feel, however, that uptake of a substrate (glucose, phosphate) by infected cells is more intimately connected with the process of virus multiplication than is the release into the medium of a metabolic end product (lactic acid).

Findings similar to these were recently reported for adenoviruses in HeLa cultures(22). Therefore the increased glucose uptake may be regarded to be involved with virus reproduction in general. Although at present it cannot be eliminated with certainty that the described changes are a reflection of non-specific symptoms of cell injury rather than viral infection, the *early* stimulation of some metabolic processes favors the assumption that a specific response is involved.

Summary. 1. A typical growth curve of poliovirus type II in human amnion cell cultures is reported. 2. During the early hours of virus multiplication and prior to onset of cytopathogenic effect human amnion cells showed a higher uptake of glucose than uninfected controls. This uptake was retarded with the active release of virus into the culture fluid. 3. Phosphorus was taken up at an increased rate by cells soon after infection, and was released back into the medium with the rise in virus titer. 4. Inhibitors of the oxidative pathways of glucose metabolism affected neither cell maintenance nor virus reproduction. It is assumed that glycolytic rather than oxidative processes are important for reproduction of poliovirus within human amnion and monkey kidney cell cultures.

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Effects of Glutamate on the Potentiating Action of Certain Ataraxics. (23651)

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(Introduced by H. R. Hulpieu)

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The potentiating activity of reserpine and of the phenothiazine group of ataraxics with barbiturates is known. These drugs probably act by different mechanisms and at different sites. The role of glutamate in cerebral metabolism, as well as its clinical usefulness, is controversial(1). Himwich has suggested that the discrepancy in the literature may be due partly to the use of the different forms of glutamate (glutamic acid, glutamic acid hydrochloride, and monosodium glutamate) and he believes that the monosodium salt is more readily absorbed(2).

Various combinations of hexobarbital, reserpine, prochlorperazine, and monosodium glutamate were administered to mice in order to study possible antagonistic, additive or potentiating activity. Monosodium glutamate moderately increased the depressant action of hexobarbital and very markedly enhanced the potentiation of reserpine on hexobarbital. This effect of monosodium glutamate was absent in prochlorperazine treated animals.

Materials and methods. Albino mice (Hartman strain) weighing 20-30 g were used only once. "Sleeping time" in the mice is reported as that time interval from the loss of, to the return of, the righting reflex following intraperitoneal injection of 100 mg/kg hexobarbital sodium (Evipal). Reserpine, prochlorperazine and hexobarbital were used as commercial ampul-forms and monosodium glutamate (MSG) as the salt. Each was diluted with distilled water and all drugs were injected intraperitoneally. Monosodium glutamate was administered one hour before the hexobarbital. Reserpine or prochlorperazine was given one-half hour before the barbiturate. A 2x3x4 factorial design was used. The 3 factors involved were: (a) sexual differences, 2 levels, ♂ and ♀; (b) ataraxics, 3 levels, 0, 5 mg/kg reserpine, 5 mg/kg prochlorperazine; (c) monosodium glutamate, 4 levels, 0, 1 g/kg, 2 g/kg and 3 g/kg. Twenty

mice were used per block; a total of 480 mice.

Results. Table I represents the results of the analysis of variance. All 3 factors (sex, ataraxics and glutamate) proved to be significant. A summary of the obtained results showing the P values of the t test is included in Table II. Administration of glutamate did not alter the effect of the barbiturate in females, but in males increased their sleeping time in higher doses (3 g/kg). Animals receiving reserpine only or glutamate and reserpine were depressed but did not lose their "righting reflex" and therefore are not included in the table. Reserpine increased the hypnotic effect of hexobarbital as has been reported by Brodie(3) and others, but pretreatment with glutamate *and* reserpine effected a sleeping time that is longer than seen in animals receiving reserpine only before hexobarbital. This is considered to be an increase in the potentiating or synergistic activity of reserpine since monosodium glutamate alone did not appreciably increase the activity of hexobarbital below doses of 3 g/kg. In female mice this increased effectiveness of reserpine was obtained only with larger doses of MSG. There was no prolongation of reserpine-barbiturate hypnosis until an amount of 3 g/kg of MSG was administered.

To determine if this unusual potentiating action of glutamate might be peculiar to reserpine or a non-specific type of synergism, prochlorperazine (Compazine), which differs markedly from reserpine in chemical configuration and pharmacological action, was administered in like manner. When prochlorperazine was substituted for reserpine there was no potentiation by glutamate.

TABLE I. F Ratios.

| Source | F ratio value | n ₁ | n ₂ | P |
|----------------|---------------|----------------|----------------|-------|
| Sex difference | 155 | 1 | 473 | <.001 |
| Tranquilizers | 302 | 2 | " | " |
| M. S. G. | 21.7 | 3 | " | " |

TABLE II. Effects of Glutamate-Reserpine Combinations on Duration of Hexobarbital-Hypnosis in Mice. 20 mice/series, 100 mg/kg of hexobarbital I.P.

| Sex | MSG, g/kg 1 hr before hexob. | Reserpine, mg/kg 1/2 hr before hexob. | Com- pazine, mg/kg 1/2 hr before hexob. | Sleeping time, \bar{X} | P values of t tests | | | | | |
|----------------|---------------------------------------|--|---|--------------------------------|-----------------------|----------------|----------------|----------------------------|--------|--------|
| | | | | | vs opposite sex | vs Ataraxie | | vs Monosodium glutamate | | |
| | | | | | | Reser- pine | Com- pazine | 1 g/kg | 2 g/kg | 3 g/kg |
| O ₃ | 0 | 0 | 0 | 17.0 | <.01 | <.001 | <.001 | n.s. | n.s. | <.01 |
| O ₃ | 1 | 0 | 0 | 17.7 | <.05 | " | " | " | " | n.s. |
| O ₃ | 2 | 0 | 0 | 20.7 | <.1 | " | " | " | " | " |
| O ₃ | 3 | 0 | 0 | 24.3 | <.001 | " | " | <.01 | " | " |
| O ₃ | 0 | 5 | 0 | 32.3 | n.s. | | " | " | <.001 | <.001 |
| O ₃ | 1 | 5 | 0 | 40.2 | <.01 | | n.s. | " | " | " |
| O ₃ | 2 | 5 | 0 | 57.2 | <.001 | | <.001 | <.001 | " | <.05 |
| O ₃ | 3 | 5 | 0 | 51.0 | <.1 | | " | " | <.05 | " |
| O ₃ | 0 | 0 | 5 | 36.2 | <.001 | n.s. | | n.s. | <.02 | n.s. |
| O ₃ | 1 | 0 | 5 | 38.2 | " | " | | " | <.01 | " |
| O ₃ | 2 | 0 | 5 | 29.5 | <.1 | <.001 | | <.01 | " | <.001 |
| O ₃ | 3 | 0 | 5 | 40.0 | <.001 | " | | n.s. | <.001 | " |
| H ₀ | 0 | 0 | 0 | 9.2 | <.01 | " | " | " | <.02 | n.s. |
| H ₀ | 1 | 0 | 0 | 12.2 | <.05 | " | " | " | n.s. | " |
| H ₀ | 2 | 0 | 0 | 15.8 | <.1 | " | <.01 | " | " | " |
| H ₀ | 3 | 0 | 0 | 13.7 | <.001 | " | <.001 | " | " | " |
| H ₀ | 0 | 5 | 0 | 31.9 | n.s. | | " | " | " | <.001 |
| H ₀ | 1 | 5 | 0 | 32.4 | <.01 | | <.01 | " | " | " |
| H ₀ | 2 | 5 | 0 | 35.3 | <.001 | | <.001 | " | " | " |
| H ₀ | 3 | 5 | 0 | 45.7 | <.1 | | " | <.001 | <.001 | " |
| H ₀ | 0 | 0 | 5 | 22.2 | <.001 | " | | n.s. | n.s. | <.1 |
| H ₀ | 1 | 0 | 5 | 24.1 | " | <.01 | | " | " | n.s. |
| H ₀ | 2 | 0 | 5 | 24.3 | <.1 | <.001 | | " | " | " |
| H ₀ | 3 | 0 | 5 | 27.1 | <.001 | " | | " | " | " |

It is interesting that there was a predominant sex-difference in response throughout our experiments. This occurred with hexobarbital alone and in animals receiving hexobarbital but pretreated with reserpine and glutamate (1 and 2 g/kg). There was no significant difference in the sexes as to response to hexobarbital when pretreated with reserpine only. Such a triple combination as we have used may have an additive activity which is not measurable as sleeping time. The general trend of results certainly indicated that there is a response to the barbiturate alone and also to the barbiturate-glutamate-reserpine combination which is dependent upon the sex of the experimental animal. Holck reports that the duration of barbiturate-induced hypnosis is different sex-wise in rats but not in mice(4). Our results indicate that studies with mice in one sex only or in un-separated sexes may result in a diversity of findings.

This relationship of sex to the potentiating action of monosodium glutamate suggested

the possibility that the toxicity might be greater in one sex. Fig. 1 is a graphic illustration of the toxicity of monosodium glutamate in mice (Winthrop Logarithmic-Probit paper). The LD₅₀ in females was 5.4 g/kg and in male mice was 6.5 g/kg. The graph emphasizes that the overall response (mortality-rate) is lower in the males at every dosage level. This is of special interest because female mice were less sensitive to the central potentiating action than were the males.

Arnold(5,6) has reported that prior administration of glutamic acid to patients delayed the hallucinatory effects of LSD (Lysergic acid diethylamide) for several hours. LSD has been shown to have an *in vitro* antiserotonin action(7). This might suggest that glutamate may have a contributory antiserotonin action as an explanation for its hyperpotentiating activity. The release of 5-hydroxytryptamine (Serotonin) is an important factor in the mechanism of activity of reserpine but this neurohormone apparently is not

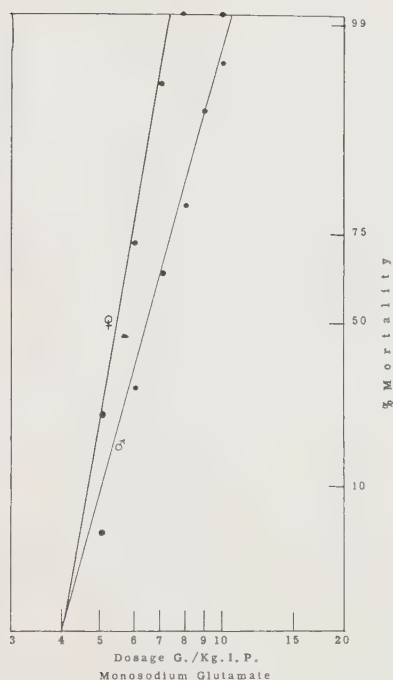


FIG. 1. Sex difference in toxicity of monosodium glutamate in mice.

implicated in the action of the phenothiazine-type of tranquilizer(8). Furthermore Lamson *et al.* has demonstrated that the administration of glutamate will reanesthetize an animal that has awakened from barbiturate hypnosis(9).

Summary. Glutamate moderately increased the mean sleeping time in mice that received hexobarbital. Reserpine markedly potenti-

ated the action of the barbiturate. Administration of glutamate prior to reserpine-hexobarbital combinations caused a further and pronounced increase in the sleeping time. This "potentiation of the potentiation" by MSG was not observed when prochlorperazine is substituted for the reserpine although prochlorperazine also increased the duration of hexobarbital-hypnosis. This suggests that the specificity of this effect of monosodium glutamate is probably dependent upon the type of tranquilizer. Regardless of which of the 2 ataraxics was used there was an overall sex difference in response to the combination of these drugs, male mice being more sensitive to the depressant drugs.

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Teratogenic Effect of Trypan Blue on the Developing Chick.* (23652)

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Gillman, Gilbert and Gillman(1) first reported the teratogenic action of trypan blue after injections into pregnant rats. Several workers have since employed this method to produce a variety of anomalies in the offspring of mice(2,3,4,5), rabbits(6,7), as well as rats

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(8,9,10). Recently Waddington and Perry (11) have reported a teratogenic action of trypan blue on amphibian embryos. The malformations reported have primarily involved the neural axis, skeleton, and heart and major vessels. Despite these relatively extensive studies, little is known of the underlying mechanism or even the site of action of the dye. It has been postulated that one or more

TABLE I. Effects of Trypan Blue on Developing Chick Embryo when Injected into Yolk-Sac and Subgerminal Cavity.

| | 24 hr yolk-sac inj.* | | 24 hr subgerminal inj.† | | 36 hr subgerminal inj.‡ | | |
|--------------------------------------|----------------------|----------------|-------------------------|----------------|-------------------------|----------------|-----------------|
| | Trypan blue | Saline control | Trypan blue | Saline control | Trypan blue | Saline control | Noninj. control |
| Total treated | 90 | 35 | 30 | 10 | 101 | 43 | 12 |
| % mortality | 74 | 20 | 73 | 40 | 45 | 11 | 1 |
| % survivors malformed | 52 | 0 | 75 | 33 | 76 | 13 | 0 |
| % survivors showing specific defects | | | | | | | |
| Rumplessness | 43 | 0 | 62 | 33 | 69 | 2 | 0 |
| Eye defects | 4 | 0 | 0 | 0 | 16 | 2 | 0 |
| Beak " | 0 | 0 | 0 | 0 | 18 | 0 | 0 |
| Gastroschisis | 0 | 0 | 25 | 0 | 5 | 2 | 0 |
| Hind limb defects | 0 | 0 | 37 | 0 | 16 | 5 | 0 |
| Others (see text) | 8 | 0 | 0 | 0 | 0 | 5 | 0 |

* Recovered on day 10.

† Recovered on day 12.

‡ Recovered on days 10 and 12.

of 3 general types of action may be involved: trypan blue may 1) produce a change in the maternal metabolism which secondarily affects the embryo, 2) cause a blockage or alteration of the placental transfer mechanism, or 3) have a direct action on the embryo. The recent work of Ferm(7) in the rabbit and Waddington and Perry(11) in amphibia has lent support to the likelihood of direct action of the dye on the embryo.

The purpose of the present investigation is to further explore the possibility of a direct action of trypan blue on an embryo, using the developing chick as a test animal.

Materials and methods. Fertile eggs of White Leghorn chickens were obtained from a commercial hatchery.† The eggs were injected under aseptic conditions with a 0.1% solution of purified trypan blue prepared in sterile saline.‡ Ninety eggs received injections of 0.1 cc into the yolk sac and 131 eggs received 0.05 cc into the subgerminal cavity during the 24th or 36th hour of incubation. Dye injected into the yolk sac was deposited in the center of the yolk with the expectation that it would float upward to underlie the developing embryo. However, the quantity of dye reaching the embryo by this method was found to be highly variable when its location was checked in hard-boiled eggs. Injections into the sub-

germinal cavity were made through a finely drawn glass needle by way of a window sawed in the shell over the embryo. The needle was introduced into the subgerminal cavity by piercing the area pellucida close to its boundary with the area opaca. Embryos were recovered on the 10th or 12th days of incubation, fixed, weighed and examined for abnormalities. Eighty-eight control eggs were treated in the same manner as the experimentals, except that sterile saline was injected. In addition, 12 control eggs were subjected to all handling procedures, including insertion of the needle, but no solution was injected.

Results. Table I summarizes the incidence as well as the types of malformations after trypan blue injection. The same types of anomalies were produced by trypan blue injection subgerminally at 24 and 36 hours, indicating that the induction of these malformations is not sharply limited in time. The marked increase in mortality in control and experimental groups treated at 24 hours, as compared with those treated at 36 hours, is attributed to the greater sensitivity of the younger embryos to the operation.

It is apparent that the most common defect produced was rumplessness (Fig. 2), seen in 88% of all abnormal experimental embryos, and also in 2 of 7 abnormal control embryos. Rumplessness occurred alone or in association with other types of malformations (Fig. 3-8). Also observed but with lesser frequency were microphthalmia, anophthalmia, crossed beak, de-

† Florida State Hatcheries, Gainesville.

‡ Obtained from Matheson, Coleman and Bell Co., Norwood, O.



All embryos pictured were recovered during 10th day of incubation.

FIG. 1. Normal embryo.

FIG. 2. Treated embryo showing a degree of the rumpless condition in which the stub of a tail remains. Note shortening of entire trunk, indicating that other than caudal vertebrae are also affected.

FIG. 3. Treated embryo with left microphthalmia, crossed beak and rumplessness.

FIG. 4. Treated embryo with left anophthalmia, crossed beak, upper beak deficient and rumplessness.

ficient beak, gastroschisis, rudimentary limbs, defective feet and malposition of the hind limbs. One case was recorded of ectopia cordis and anencephaly. Not regarded as malformations were 5 embryos with generalized edema and 4 with retarded growth. The defects in 7 abnormal control embryos included 2 cases of rumplessness, 1 of exophthalmia, 1 of gastroschisis, 1 of malposition of the hind limb, 1 of defective feet and 2 of spina bifida. The abnormalities common to both the experimental and control embryos were rump-

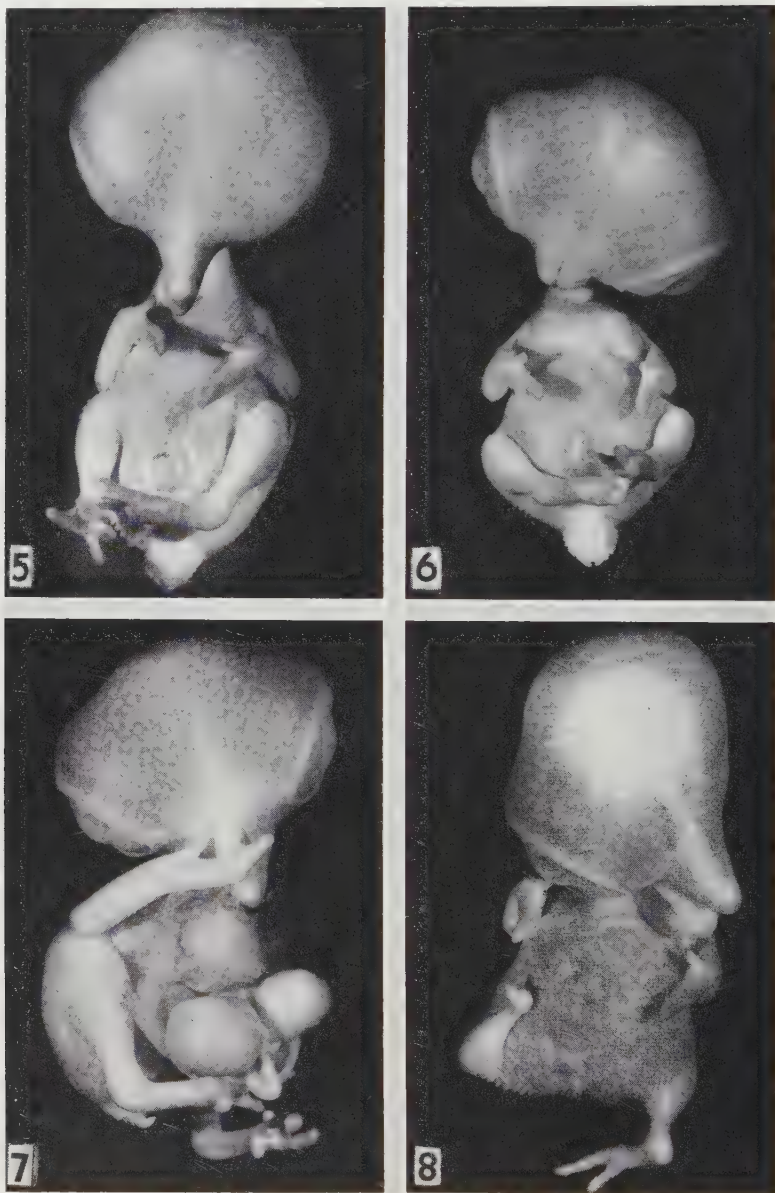
lessness, gastroschisis and skeletal defects of the hind limbs.

All experimental and control animals were weighed after fixation. No significant difference in weight was noted.

Discussion. Our results indicate that trypan blue can act upon the embryo of the developing hen's egg to produce abnormalities similar to those already described in the rat. This is particularly true in the case of ocular and vertebral (rumpless) defects. Perhaps the most significant aspect of the results is

the demonstration that no placental organ or maternal reactions are necessary for production of malformations with trypan blue. These observations together with those of Waddington and Perry(11) on frogs indicate

a direct action of trypan blue on developing embryonic systems. Unfortunately, however, Waddington and Perry do not report control animals for their amphibian experiments. We have observed that decapsulation of frog



All embryos pictured were recovered during 10th day of incubation.

FIG. 5. Normal embryo.

FIG. 6. Treated embryo with crossed beak and deficient upper beak. Size and appearance are more nearly those of an 8-day than a 10-day embryo and indicate considerable growth retardation.

FIG. 7. Treated embryo with ectopia cordis, gastroschisis and rumplessness.

FIG. 8. Treated embryo with left anophthalmia, crossed beak, rudimentary right leg, rumplessness, and malposition of left leg with ectrodactyly.

TABLE II. Comparison of Incidence of Spontaneous Malformations Occurring in White Leghorn Chicks with Incidence of Malformations Produced by Trypan Blue Injected at 36 Hr into the Subgerminal Cavity.

| Defect | Spontaneous,* % | Trypan blue induced, % in survivors |
|-----------------------------------|-----------------|-------------------------------------|
| Rumplessness | 1.00 | 69.0 |
| Anophthalmia | .48 | 3.6 |
| Microphthalmia | .28 | 3.6 |
| Cross beak | .28 | 9.1 |
| Upper beak rudimentary or missing | .07 | 9.1 |
| % total No. of deformed chicks | .14 | 70.0 |

* Defect and spontaneous incidence selected from a Table by Landauer and Baumann(12).

eggs, as practiced in their investigation, can produce many of the abnormalities attributed to the action of the dye.

A comparison of the incidence of malformations produced in the present study with those occurring spontaneously in the White Leghorn chicken yields interesting similarities and differences (Table II). Many of the same types of defects occurred in both groups but it is noteworthy that other abnormalities did not. The incidence of certain defects that occur spontaneously appears to be greatly increased by application of the dye. Landauer and Baumann(12) report a seasonal variation in frequency of appearance of spontaneous rumplessness with the incidence higher in the late spring and summer than at other times of the year. To what extent such seasonal variation has affected the results reported here is not known, but most of the injections were made during the late spring and early summer.

Considerable variation was observed in the reaction of different batches of eggs to the same type of treatment. Eggs were injected in lots of 2 to 3 dozen at a time. In all injection lots rumplessness was the most common malformation but the concomitant abnormalities varied noticeably in type and incidence from lot to lot of eggs. A possible source of such variation may be the fact that the eggs were obtained from different flocks and, therefore, were not genetically consistent. In the same flock different hens are known to produce offspring with varying tendency toward

such defects as rumplessness, whether spontaneously occurring or experimentally induced (12).

The production of rumplessness and defects of the appendicular skeleton, beak, and eyes in the chick is by no means unique to trypan blue. Landauer(13) has shown that many chemical agents produce similar defects when introduced into the yolk sac. The underlying mechanism of action is not known for any of the several agents. Landauer has postulated interference with metabolic functions at the cellular level but no adequate explanation is available for the peculiar susceptibility of such tissues as the tail, for example.

Summary. 1. Eggs of White Leghorn chickens were injected with saline solution of trypan blue into the yolk sac at 24 hours and into the subgerminal cavity at 24 and 36 hours. 2. Yolk sac injections caused a mortality of 74% and resulted in malformation of 52% of the survivors on the 10th day. 3. Subgerminal cavity injections caused 73% mortality at 24 hours and 45% mortality at 36 hours. Of the surviving embryos at 10 and 12 days, approximately 75% in each group were abnormal. 4. The abnormalities were essentially the same after both types of treatment and included, in order of incidence, rumplessness, hind limb defects, beak defects, eye defects, and gastroschisis. 5. It is concluded that, at least in the chick, trypan blue is capable of teratogenic action directly on the embryo and does not require as an intermediary site the maternal organism or a placenta.

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An Effect of Pyridine-2-Aldoxime Methiodide (2-PAM) on Cholinesterase at Motor End-Plates. (23653)

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Certain oximes have an effect on the end-plate potential of isolated nerve-muscle preparations(1,2). Therefore, the following experiments were done to study the action of the oxime, pyridine-2-aldoxime methiodide (2-PAM), on cholinesterase at motor end-plates in striated muscle.

Materials and methods. The oxime 2-PAM was dissolved in concentrations of $7.6 \times 10^{-3} M$ and $1 \times 10^{-3} M$ either in frog Ringer solution or in incubation medium for the Koelle technic(3) immediately before use. Tetraethylpyrophosphate (TEPP) was used in frog Ringer solution in approximately $1.5 \times 10^{-5} M$ concentration for inactivation of cholinesterase (ChE). The *iliofibularis* muscle of *R. pipiens* was freed of surrounding connective tissue and separated into small portions lengthwise. Thus many of the muscle fibers and their motor end-plates were in intimate contact with the solutions. During each of

the two 30-minute exposure periods the fibers were immersed in Ringer solution or in a solution of 2-PAM or TEPP in the pH range indicated in Table I and at temperatures of 24° - $28^{\circ}C$. These preparations were rinsed in Ringer solution after each exposure period. Each was then transferred to the incubation medium containing acetylthiocholine as substrate either with or without 2-PAM and incubated for 30 minutes at 24° - $28^{\circ}C$. The tissues were then mounted in glycerine on slides and examined microscopically. ChE activity was assessed by the relative amounts of copper sulfide (CuS) precipitated on the subneural apparatus and recorded quasi-quantitatively; ++++ indicating maximum activity and 0, too little activity to be detectable by this method.

Results. Photographs of representative end-plate areas showing various degrees of ChE activity are illustrated in Fig. 1. The

TABLE I. Histochemical Demonstration of Interactions between 2-PAM, TEPP and ChE in Frog Muscle.

| Exposure periods,* pH 7.75-7.10 | | Incubation period pH 5.35-5.30 | ChE activity |
|---------------------------------|----------------------|-----------------------------------|-----------------|
| 30 min. | 30 min. | 30 min. | |
| 2-PAM = $7.6 \times 10^{-3} M$ | | | |
| (1) Ringer sol | Ringer sol | Medium + 2-PAM | + |
| (2) 2-PAM in Ringer sol† | 2-PAM in Ringer sol† | Medium only | +++ |
| (3) TEPP <i>Idem</i> | Ringer sol | Medium " | 0 |
| (4) TEPP " | 2-PAM in Ringer sol | Medium " | +++ |
| (5) TEPP " | 2-PAM <i>Idem</i> | Medium + 2-PAM | + |
| (6) 2-PAM " | TEPP " | Medium only | 0 |
| 2-PAM = $1 \times 10^{-3} M$ | | | |
| (7) Ringer sol | Ringer sol | Medium + 2-PAM | +++ |
| (8) TEPP in Ringer sol | 2-PAM in Ringer sol | Medium <i>Idem</i> | ++ |
| (9) 2-PAM <i>Idem</i> | TEPP <i>Idem</i> | Medium only | 0 |

* After each exposure period the preparation was rinsed in Ringer solution.

† These exposures were made at pH 5.35-5.30 also with identical results.

‡ Control preparation, incubated without 2-PAM, showed maximum (++++) deposition of CuS.

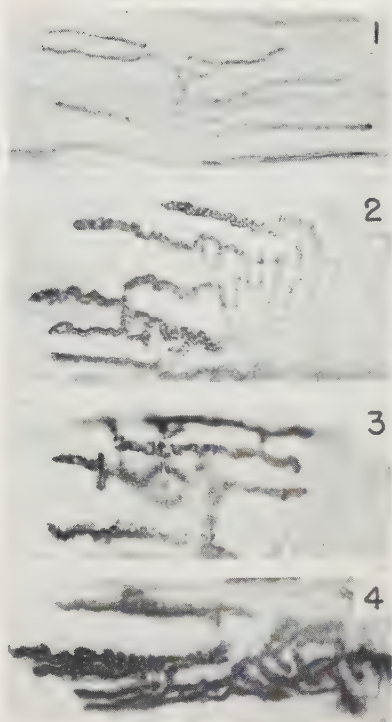


FIG. 1. Subneural apparatus of motor end-plate in *iliofibularis* muscle of frogs, stained for cholinesterase by a modified Koelle technique. ChE activity, as assessed by CuS deposit is illustrated as follows: 1, +; 2, ++; 3, +++; 4, ++++. Muscles were incubated for 30 min; magnification 275 X.

enzyme is localized in the sub-neural apparatus.

As shown in line 1 of Table I, if 2-PAM was present during incubation of tissue at pH 5.35 to 5.30 in a concentration of $7.6 \times 10^{-3} M$, there was decided inhibition of ChE activity. This effect was readily reversible (line 2) where exposure to 2-PAM in Ringer solution prior to but not during the incubation period diminished ChE activity only slightly. Furthermore, if the ChE was inactivated by TEPP, as shown in line 3, subsequent exposure to 2-PAM restored much of the activity (line 4). However, incubation with 2-PAM of a preparation previously exposed to both TEPP and 2-PAM showed less total ChE activity, as shown in line 5. Exposure of tissue to 2-PAM prior to but not during exposure to TEPP did not protect the ChE from the latter, as shown in line 6. When these experiments were repeated using 2-PAM

in a concentration of $1 \times 10^{-3} M$, the ChE depressing effect of 2-PAM during the incubation period was less striking; compare line 7 with line 1. Concomitantly, the reactivating effect of 2-PAM was greater in the lower of the 2 concentrations even though it was added to the incubation medium containing tissue previously exposed to both TEPP and 2-PAM; compare line 8 with line 5. Presumably this was the result of less of the direct inhibiting effect of 2-PAM on ChE since the oxime was present in lower concentration. Again, at this concentration, 2-PAM gave no protection to ChE against later exposure to TEPP, line 9.

Discussion. Although the pH of the solutions used in the exposure periods was kept between 7.75 and 7.10, the pH for the incubation period was lowered to between 5.35 and 5.30. By lowering the pH below the usual 6.0 of the Koelle formula, it was possible to hold to a minimum the reaction between the substrate, acetylthiocholine, and 2-PAM when it was added to the incubation medium(4). Both the reaction between enzyme and substrate and the competing reaction between oxime and substrate are pH dependent. The diminution in ChE activity accompanying this lowering of pH was compensated by a slightly longer period of incubation (30 instead of 25 minutes) at pH 5.3.

Previous *in vitro* studies of the action of oximes on ChE in tissues have been based on the use of homogenates of tissue, red blood cell suspensions, whole muscle and nerve-muscle preparations(5-9). In the present experiments fresh muscle fibers were employed. A concentration of 2-PAM that is only slightly inhibitory for ChE, as demonstrated with this technic, has a reactivating effect on previously inactivated enzyme. In a higher concentration, 2-PAM had a marked inhibitory effect on ChE. The oxime effect was reversible. After rinsing preparations previously exposed to 2-PAM, no protection to ChE from the effect of subsequent exposure to TEPP was demonstrated. In the presence of the higher concentration of 2-PAM, ChE previously inactivated with TEPP showed less restoration of function. This may represent a supplement-

tary inhibiting effect of 2-PAM. If such an interpretation is correct, it seems logical to assume that 2 aspects of activity of 2-PAM were demonstrated in the preparation described by line 5 of Table I. First, TEPP-inactivated ChE was reactivated and subsequently it was inhibited by the oxime itself.

Conclusion. 2-PAM in a high concentration *in vitro* had a dual effect on motor end-plate ChE previously inactivated with TEPP. The oxime reactivated the enzyme and, if allowed to remain in contact with the preparation, inhibited the reactivated enzyme. The effect of 2-PAM was reversible and after the removal of 2-PAM no protection to ChE from the later exposure to TEPP was demonstrated.

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Relationship of Dietary Dienoic Acid Content to That in Mouse Carcass Fat.* (23654)

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The investigation here reported was suggested by results obtained in a previous study (1) that showed an increase in carcass fat dienoic acids of animals fed rigorously extracted soybean oil meal. The results of the study indicated that the increased carcass fat dienoic acids were not the result of residual dienoic acids in the meal. Hence, work attempting to isolate the causative factor was initiated. The fractionation work revealed that the ether-extracted soybean meal contained sufficient dienoic acids, which could be removed by methanol extraction, to account for the increased carcass fat levels of these acids. During these experiments it became necessary to investigate the nature of the response curve of carcass fat dienoic acids to dietary dienoic acids, since little information is available about the quantitative aspects of this relationship especially at low dietary levels.

Methods. Weanling albino mice obtained from the North Carolina State Laboratory of Hygiene were used in these studies. They were housed individually on wire screens and received *ad libitum* an egg albumin-starch purified diet similar to diet 13 of the previous studies(1). Each animal was parenterally given 0.01 mg biotin twice weekly. The various adjuncts that were tested for their capacity to alter the carcass fat dienoic acid content were added at the expense of starch. Each experiment was of 3 weeks duration with the average of 6 replications reported. At the completion of the experiment the mice were gassed, skinned, decapitated, and the entire gastrointestinal tract removed. The mouse carcass was then placed into a 30-ml beaker, minced with scissors and dehydrated by freeze drying. The dry carcass was ground with about 1 g of Super-Cel and about 2 g of anhydrous sodium sulfate. This mixture was placed into a coarse alundum crucible and extracted with anhydrous ether for 12-18 hrs in a Pickel extractor. After evaporation of the

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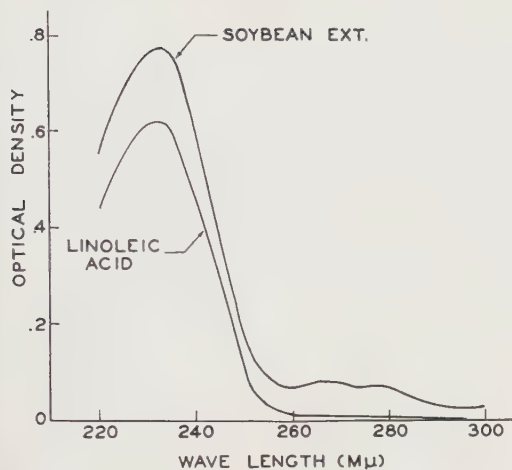


FIG. 1. Absorption spectrum of isomerized fatty acids from soybean extract and linoleic acid at concentrations of 18 and 7 mg/l, respectively.

ether, the polyunsaturated acids in the carcass fat were determined by alkaline isomerization(2). Soybean meal extracts were prepared in the following manner: Commercial solvent-extracted soybean meal was continuously extracted with ether for 48 hrs. The ether extracted meal was then continuously extracted with methanol at 65°C for 48 hrs, and the methanol extract dried on starch after removal of the solvent. An ether-soluble portion of the methanol extract was prepared by extracting the methanol extract, after adjusting the pH to 4, with ether in a continuous liquid-liquid extractor for 48 hrs.

Results. 1. Factor studies. Fractionation work aimed toward isolation of the factor suggested by previous work revealed that the factor could be extracted from the meal with methanol, and further that continuous ether extraction of the methanol-soluble portion removed all the activity. Since these results would be consistent with the presence of residual dienoic acids in the ether extracted soybean meal, this possibility was investigated more rigorously than had been done in the previous studies(1).

The ether-soluble fraction of the methanol extract was saponified with 10% alcoholic potassium hydroxide and the fatty acids obtained divided into 2 portions. One of these was subjected to alkaline isomerization and the adsorption spectrum compared with an

authentic sample of linoleic acid. The spectral curve, Fig. 1, of the acids closely resembles that of the isomerized linoleic acid. The two peaks observed in the region of 270 mμ are characteristic of isomerized trienoic acids. Chromatography of the second portion according to the procedure of Simmons and Quackenbush(3) revealed that both dienoic and trienoic acids were present.†

Although previous results(1) indicated that a solvent extract of acid-hydrolyzed soybean meal failed to increase the carcass fat dienoic acids, the detection of polyunsaturated acids in the soybean meal extracts necessitated repetition of this experiment. The lipids from 750 g of ether-extracted soybean meal were obtained by ether and petroleum ether extraction after acid hydrolysis according to the AOAC procedure for the determination of fat in cereal products(4). After removal of the solvents under reduced pressure, the lipid was dissolved in ethanol and dried on starch at room temperature. Contrary to the previous results from this type of preparation(1), the lipids equivalent to 75% soybean meal increased the carcass fat dienoic acids from 2.7% to 6.1%

2. Response curve studies. Prior to the detection of the presence of polyunsaturated acids in the soybean meal extracts, attempts were made to develop a quantitative assay for the substance in these extracts that increased the dienoic acid content of carcass fat. In the previous work(1), the greatest relative response was obtained when no dietary fat was added to the basal diet. Therefore, a basal diet without added fat was adopted, and this was supplemented with graded levels of the soybean meal methanol extract. The results of two such experiments, Fig. 2, show that although the lower part of the response curve is linear, at the highest level of soybean meal extract the response curve becomes curvilinear. When the logarithm of intake is plotted against the logarithm of carcass fat dienoic acid level, a straight line, which includes the high level, is obtained.

To further verify the response curve and to

† The authors are indebted to Dr. R. O. Simmons for the chromatographic analysis.

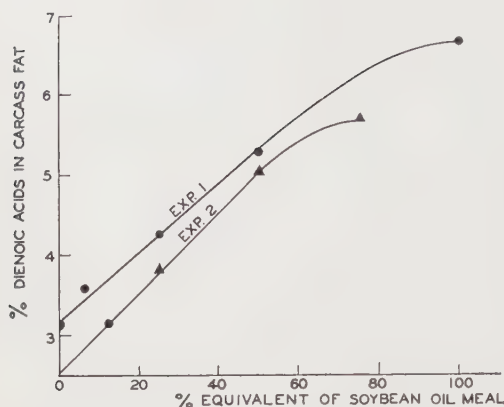


FIG. 2. Effect of feeding methanol extract of soybean meal on dienoic acid content of mouse carcass fat.

extend its range to 400% equivalent soybean meal a third experiment was conducted. The organic solids of the methanol extract as determined by the method of Johnson(5) using a sucrose standard was equivalent to 140 mg/g of soybean meal. Thus, if the extract were fed equivalent to 400% soybean meal, at least 56% of the diet would be composed of extract solids. Since the ether-soluble extract equivalent to 400% soybean meal would contain only 13.6% organic solids, this ether extract was used instead of the crude methanol extract. To serve as a basis of comparison, graded levels of soybean oil also were fed as a source of dietary dienoic acids. As was observed previously, a curvilinear response was obtained, Fig. 3, and a log-log plot resulted in a straight line up to an equivalent of 200% soybean meal and a dietary level of dienoic acids of 0.8% as soybean oil. However, when either 400% soybean-meal equivalent or 1.6% dienoic acids as soybean oil was fed, the carcass fat dienoic acids were distinctly higher than would be expected by extrapolation of the curve.

Discussion. In view of the similarity of the response curves from the ether-extracted soybean-meal fractions to that from soybean oil, the chemical properties of the active principle and the demonstrated presence of dienoic acids in both the ether-extracted soybean meal and its active fractions, it is apparent that the increased carcass fat dienoic acid level obtained by feeding the ether-extracted soy-

bean meal is a result of residual amounts of dienoic acids in the meal. From the response curve of Fig. 3, it can be calculated that the meals or meal extracts should contain approximately 0.4% dienoic acids. This is in good agreement with the 0.4% calculated from feeding the fat-soluble portion of the acid hydrolyzed meal and 0.38% and 0.47% found by analysis of the extract by alkaline isomerization and chromatography, respectively.

In the previous work(1), the lipid fraction of acid hydrolyzed meal failed to produce a response. It is possible that the dienoic acids were oxidized during the course of diet preparation or storage, but there is no evidence at hand to support this.

It is apparent from an examination of the response curves obtained when graded levels of dienoic acids are fed either as soybean oil or soybean meal extracts that up to a level of 0.8% dietary dienoic acids a curvilinear response curve is obtained. Within the range of 0.2% and 0.8% dietary dienoic acids, plotting the logarithm of dietary level against the logarithm of carcass level results in a straight line. The log-log relationship would be particularly useful in the determination of biological availability of dietary dienoic acids. At dietary levels greater than 0.8%, a greater proportion of the ingested dienoic acids is found in the carcass fat.

Ellis *et al.*(6) fed graded levels of cottonseed oil to swine and measured the linoleic acid content of the carcass fat. If these data are graphed, an upward curvilinear response curve is obtained. Since the lowest level of

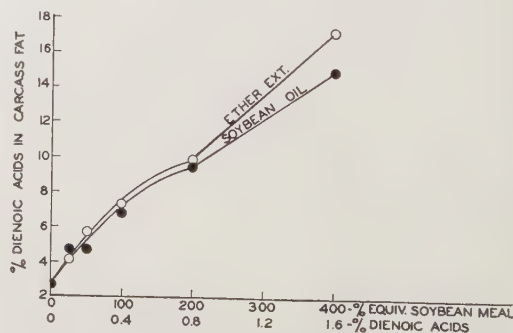


FIG. 3. Effect of feeding ether soluble portion of a methanol extract of soybean meal and dienoic acids as soybean oil on carcass-fat dienoic acid level.

cottonseed oil fed was 3%, these data are not in conflict with the observations reported herein. In fact, the disproportionately increased dienoic acid deposition obtained with the highest level (Fig. 3) suggests a similar upward curvilinear trend. Mead *et al.*(7) have shown that a large proportion of the ingested linoleic acid is rapidly oxidized. Conversely, from the prolonged time required for the depletion of linoleic acid from animal tissues, that deposited is relatively metabolically inert.

It would seem probable that as the dietary level of dienoic acids changes the ratio of the amount oxidized to that deposited also changes. The data presented herein coupled with that of Ellis *et al.*(6) may be interpreted to indicate at least 3 such changes. At dietary intakes up to about 0.2% dienoic acids, the ratio of dienoic acids oxidized to that deposited remains constant. Between about 0.2%-0.8% a larger proportion of the ingested dienoic acids is oxidized, and as the levels increase above 0.8%, an increasingly larger fraction of the ingested dienoic acids is deposited. The foregoing considerations are, of course, largely speculative and considerably more evidence is required for confirmation.

Summary. (1) Ether extracted soybean oil meal has been found to contain sufficient residual dienoic acids to account for the previously observed increased carcass fat dienoic

acids of animals that ingest the meal. (2) Studies were conducted, with mice, on the carcass-fat levels of dienoic acids obtained as the dietary-dienoic-acid levels varied from 0 to 1.6%. Between the dietary levels of 0.1% and 0.8% dienoic acids, a curvilinear response curve was obtained and became linear when the logarithm of dietary intake was plotted against the logarithm of carcass fat level. At a dietary level of 1.6% dienoic acids, a disproportionately higher dienoic acid level was found in the carcass fat.

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Effect of Dibenamine and Pyribenzamine on Hypothermia of Chlorpromazine. (23655)

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Tranquilizing drugs such as reserpine and chlorpromazine injected into an animal are reported to release a variety of substances which independently have a diversity of actions. These substances are 5 hydroxytryptamine(1), some sympathetic neurohumoral agent(2,3,4), and possibly histamine.* All

these substances have a common effect which is that of lowering the body temperature(5,6,7,8). The purpose of this study was to determine the effect on body temperature of certain drugs which are known antagonists to these products released by chlorpromazine. The substances used were dibenamine, which antagonizes the edema producing effect of 5-

* Unpublished.

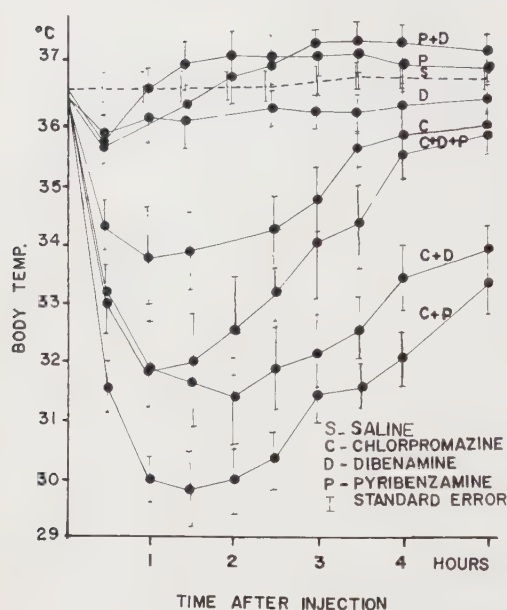


FIG. 1. Effect of intraperitoneal injections of chlorpromazine (8.5 mg/kg), dibenamine (21 mg/kg) and pyribenzamine (10.5 mg/kg) on body temperature of rats. (The standard error at 0 time is between 0.1 and 0.2°C for all groups.)

hydroxytryptamine(9) and has well known potent adrenergic blocking properties, and pyribenzamine, one of the antihistamines.

Methods. Eight groups of 6 white male rats, weighing 120 g, were used. These groups, which were all injected at the same time and on the same day, were treated respectively with: 1) chlorpromazine (8.5 mg/kg) (CPZ); 2) CPZ (8.5 mg/kg) + dibenamine (21 mg/kg); 3) CPZ (8.5 mg/kg) + pyribenzamine (10.5 mg/kg); 4) CPZ + dibenamine (21 mg/kg) + pyribenzamine (10.5 mg/kg); 5) dibenamine (21 mg/kg); 6) pyribenzamine (10.5 mg/kg); 7) dibenamine (21 mg/kg) + pyribenzamine (10.5 mg/kg) and 8) saline (8.5 mg/kg). Each drug was injected intraperitoneally and prepared in such concentration that the injected volume was 0.1 ml. The following fluid was used to dissolve 100 mg of dibenamine: 24.2 ml ethanol, 24.2 propylene glycol ml and 0.1 ml concn. HCl. Prior to its use, this solution was diluted with saline to the proper concentration. Body temperature was measured with a thermocouple inserted through the anus to a depth of 40 mm. The animals were kept

in a room maintained at 72° F.

Results. Fig. 1 shows that dibenamine and pyribenzamine injected alone or simultaneously will cause a drop of approximately 1°C in body temperature. A reversal of this effect is observed 1 hour after injection although the dibenamine group always remains significantly lower than the other 2 groups. The usual hypothermic effect of chlorpromazine, with a maximum intensity between 1 and 2 hours, is observed. Both pyribenzamine and dibenamine independently potentiate this hypothermic effect of chlorpromazine to an extent far greater than could be predicted from results obtained with these drugs alone. When dibenamine and pyribenzamine are added to chlorpromazine simultaneously, the intensity of the response is smaller and the recovery faster than could be anticipated on the basis of additive action.

Discussion. The possibility that chlorpromazine induces hypothermia by release of substances such as 5 hydroxytryptamine, some neurohumoral agent or histamine(5,6,7,8), suggested that antagonists to the released substances might alleviate hypothermic effects of chlorpromazine. Experimental evidence, however, indicates that dibenamine and pyribenzamine potentiate rather than antagonize chlorpromazine. Evidence of potentiation between histamine and an antihistaminic with respect to gastric secretion has been given(10,11,12). It has been suggested that such an effect may be obtained if the antagonist competes with the active substance in some specific tissue(13). This hypothesis would suggest that antihistaminics increase blood levels of histamine and thus alter temperature regulating centers. The same may be true of dibenamine with regard to adrenaline, with the result that the level of adrenaline or some neurohumoral agent in circulation is increased. This increase would then result in a decreased oxygen consumption since adrenaline, while it increases the heat production at 94°F, will actually decrease the metabolic exchanges when injected into an animal kept at 72°F(8).

Summary. The hypothermic effect of dibenamine and pyribenzamine was observed

but was found to be of small intensity and short duration compared to that of chlorpromazine. Individually these drugs were found to potentiate the hypothermic effect of chlorpromazine to an extent far greater than could be predicted. Indeed, whereas dibenamine or pyribenzamine caused a drop in rectal temperature of less than 1°C, these drugs injected individually with chlorpromazine potentiated the effect of chlorpromazine on body temperature by 3 to 4°C. However, dibenamine and pyribenzamine antagonize one another when given simultaneously to animals treated with chlorpromazine; this antagonism further illustrates the complexity of action of these drugs.

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Synergism of Amines and Antagonism of Reserpine to Morphine Analgesia. (23656)

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It has been demonstrated(1) that reserpine antagonizes the analgetic effect of morphine in mice. Since reserpine is known to deplete tissues of 5-hydroxytryptamine(2) and catecholamines(3), experiments were performed to determine whether morphine analgesia was influenced by administration of various indolalkylamines and catecholamines and some of their derivatives.

Methods. Seven hundred sixty (760) white mice (Webster strain) with an average weight of 20 g were used. The tail of each mouse was subjected to a painful heat stimulus according to the technic of Gross(4) and the reaction time for the occurrence of the tail flick measured. The method has been previously described(1). The effects of morphine sul-

fate were studied alone and combined with reserpine phosphate, isoreserpine, 5-hydroxytryptamine, 5-hydroxytryptophane, 5-hydroxyindoleacetic acid, tryptophane, tryptamine, epinephrine, amphetamine, mescaline, iproniazid and choline-p-tolyl-ether. In addition, the influence of reserpine was observed on codeine and meperidine analgesia. All drugs were injected subcutaneously and dissolved in distilled water with the exception of isoreserpine which was dissolved in N, N-dimethylacetamide. The significance of the results was evaluated according to the ranking method described by Moroney(5)*.

Results. Table I illustrates and confirms the previous finding that reserpine antagonizes the morphine-induced prolongation of the reaction time in the tail flick test when

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TABLE I. Antagonism of Reserpine to Analgesics (10 Mice/Group).

| | Dose, mg/kg | Avg reaction time in sec. | | | |
|-------------------------|----------------|---------------------------|------------|------------|------------|
| | | Control | 30 min. | 60 min. | 120 min. |
| Morphine sulfate | 5 | 3.9 | 7.4 | 6.4 | 4.9 |
| Morphine sulfate | 5 | | | | |
| 0 hr after reserpine | 2.5 | 4.1 | 7.6 (n.s.) | 7.4 (n.s.) | 5.0 (n.s.) |
| 2 hr " " | 2.5 | 4.3 | 5.3 (<5%) | 5.1 (") | 4.5 (") |
| 4 hr " " | 2.5 | 4.0 | 4.8 (") | 4.8 (") | 4.4 (") |
| 16 hr " " | 2.5 | 3.9 | 4.4 (<1%) | 4.5 (<5%) | 4.0 (") |
| Morphine sulfate | 10 | 4.3 | 8.6 | 9.9 | 7.5 |
| Morphine sulfate | 10 | | | | |
| 2 hr after isoreserpine | 10 | 3.0 | 8.3 (n.s.) | 8.8 (n.s.) | 7.7 (n.s.) |
| Codeine | 50 | 4.2 | 9.7 | 5.4 | 5.1 |
| Codeine | 50 | | | | |
| 2 hr after reserpine | 10 | 3.8 | 4.7 (<1%) | 4.5 (n.s.) | 3.5 (n.s.) |
| Meperidine | 50 | 3.9 | 8.9 | 7.8 | 5.6 |
| Meperidine | 50 | | | | |
| 2 hr after reserpine | 10 | 3.8 | 5.3 (<1%) | 4.9 (<1%) | 4.2 (n.s.) |

n.s. = not significant.

injected 2 to 16 hours prior to the experiment. Similarly reserpine antagonized the analgetic effect of codeine and meperidine, whereas iso-reserpine which lacks reserpine-like pharmacodynamic activity(6) failed to have this effect.

Five-hydroxytryptamine (5-10 mg/kg), tryptamine (100 mg/kg) and 5-hydroxytryptophane (30-400 mg/kg) enhanced and prolonged the analgetic effect of morphine markedly (Fig. 1). Very high doses of 5-hydroxytryptamine without morphine (20 mg/kg) were well tolerated and produced also a pro-

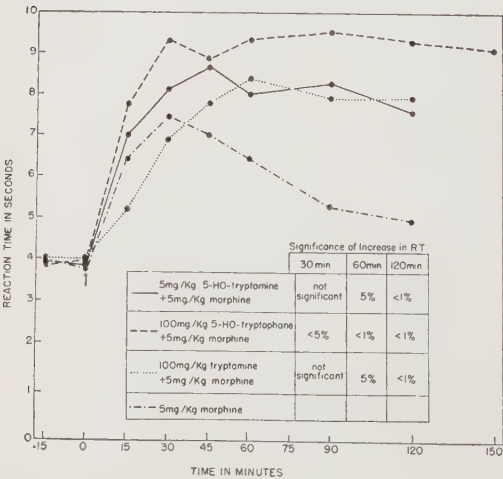


FIG. 1. Influence of 5-hydroxytryptamine and derivatives on morphine analgesia in mice.

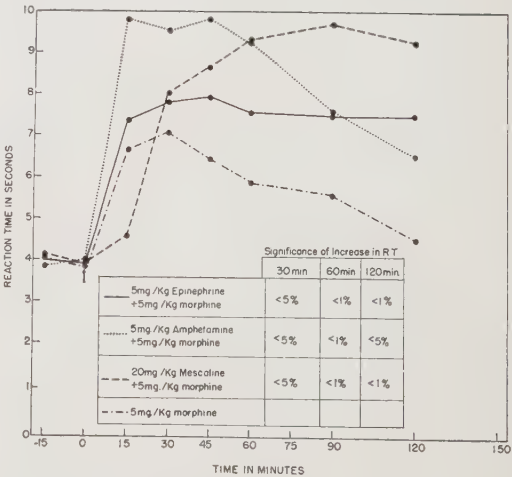


FIG. 2. Effect of some amines on morphine analgesia in mice.

longation of the reaction time. Five-hydroxytryptophane by itself however did not alter the reaction time even in very large amounts (400 mg/kg). Tryptophane and 5-hydroxyindoleacetic acid (a degradation product of 5-hydroxytryptamine) did not affect the analgetic effect of morphine at a dose of 100 mg/kg.

Amphetamine (3-5 mg/kg), mescaline (10-50 mg/kg) and to a lesser extent high doses of epinephrine (2-5 mg/kg) potentiated and prolonged morphine analgesia (Fig. 2). Epinephrine and norepinephrine in doses of

TABLE II. Prevention of Reserpine-Induced Morphine Antagonism by Iproniazid (10 Mice per Group).

| | Dose, mg/kg | Avg reaction time in sec. | | | |
|--|----------------|---------------------------|------------|------------|------------|
| | | Control | 30 min. | 60 min. | 120 min. |
| Morphine sulfate | 5 | 4.1 | 8.3 | 7.5 | 5.4 |
| Morphine sulfate 2 hr after reserpine | 2.5 | 3.8 (n.s.) | 7.0 (5%) | 6.0 (5%) | 4.9 (n.s.) |
| Morphine sulfate 6 hr after iproniazid + 2 hr after reserpine | 100.0 2.5 | 4.0 (") | 9.0 (n.s.) | 6.9 (n.s.) | 5.3 (") |
| Morphine sulfate 18 hr after iproniazid + 2 hr after reserpine | 100.0 2.5 | | | | |

n.s. = not significant.

less than 2 mg/kg did not alter morphine analgesia. Surprisingly, neither iproniazid (100 mg/kg) nor choline-p-tolyl-ether (100 mg/kg) enhanced the analgesic effect of morphine significantly, no matter whether they were injected simultaneously or several hours before morphine administration. However, reserpine no longer antagonized morphine in mice which were pretreated with iproniazid (Table II).

Discussion. The previous finding(1) that reaction time to the mouse tail flick prolonged by morphine is shortened by reserpine has been confirmed. In addition, reserpine also was shown to antagonize the analgetic effect of codeine and meperidine. Tripod and Gross (7) recently reported a synergistic effect of reserpine on morphine analgesia in the same test. The discrepancy in these results cannot be explained at present. As can be seen from Table I, simultaneous administration of reserpine and morphine did not alter morphine analgesia significantly whereas morphine administration 2-16 hours following reserpine showed a progressive diminution of the analgetic effect.

This latter observation raises the question whether the reserpine antagonism of morphine analgesia is linked with the concentration of indolalkylamines or catecholamines in the central nervous system.

From the investigations of Pletscher *et al.* (2) and Holzbauer and Vogt(3) it is known that reserpine depletes body tissues of their 5-hydroxytryptamine and catecholamine content. If this process plays a role in the

mechanism of morphine antagonism of reserpine, an increased level of these substances might be expected to potentiate the analgetic effect of morphine. This is indeed the case for 5-hydroxytryptamine and its precursor, 5-hydroxytryptophane. Five-hydroxytryptamine itself increases the reaction time to a heat stimulus when given in very high doses.

Epinephrine in high doses (2 mg/kg and more) has a distinct potentiating effect, a finding which confirms the report of Hurst and Davies(8). Gross and Kaufmann(9) reported that epinephrine in the dose range of 0.05 to 0.25 mg/kg did not influence morphine analgesia. In this connection it is interesting to note that morphine has been found to release epinephrine(10) and norepinephrine(11) but not 5-hydroxytryptamine.

Choline-p-tolyl-ether and iproniazid, both inhibitors of amino oxydase(12,13) failed to potentiate morphine analgesia. However, if the destruction of 5-hydroxytryptamine and catecholamines is prevented by administration of iproniazid, reserpine no longer antagonizes the analgetic effect of morphine. This indicates that the reserpine-induced morphine antagonism is likely to be due to the depletion of 5-hydroxytryptamine and/or catecholamines.

The result that several other amines such as amphetamine(14), tryptamine and mescaline enhance and prolong the analgetic effect of morphine suggests that the morphine potentiation produced by 5-hydroxytryptamine is not specific and that another mechanism of morphine synergism must be of importance.

Cholinergic mechanisms have attracted attention since morphine action is synergistically influenced by neostigmin(15) and ibogaine(16), both reported to be cholinesterase inhibitors. According to several authors(17, 18) morphine itself inhibits cholinesterase activity markedly. However, the point of view that the mechanism underlying morphine synergism is linked to cholinesterase inhibition cannot be maintained since pilocarpine and carbamylcholine both of which do not affect cholinesterase are active synergists, whereas tetraethyl pyrophosphate, a strong inhibitor, is inactive(19). In addition, no parallelism exists between analgesic action and the degree of cholinesterase inhibition(20). The weak cholinesterase inhibitory activity of 5-hydroxytryptamine and tryptamine(21) can therefore hardly be used to explain the morphine synergism of these agents.

It seems more appropriate to look for a more common factor involved in morphine potentiation. It has been demonstrated that diethylaminoethyl - diphenyl - propyl - acetate which enhances morphine analgesia(22) and prolongs hexobarbital sleeping time(23) inhibits rate of metabolism of hexobarbital and retards demethylation of meperidine thereby prolonging their action(24,25,26). A similar mechanism might play a role in the case of morphine synergism, induced by a variety of drugs.

Summary. (1) Reserpine antagonizes the analgesia in mice induced by morphine, meperidine and codeine. (2) Five-hydroxytryptophane, 5-hydroxytryptamine, tryptamine, amphetamine, mescaline and epinephrine prolong and enhance morphine analgesia whereas iproniazid and choline-p-tolyl-ether fail to do so. (3) Possible mechanisms involved in the synergistic action of amines and the antagonistic action of reserpine on morphine analgesia are discussed.

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Limited Multiplication of *Mycobacterium lepraemurium* in Cell Cultures. (23657)

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Since the demonstration of *M. lepraemurium* and its recognition as the causative agent of rat leprosy (1,2), this intracellular parasite has been an object of unusual interest with the problem of human leprosy. Like the human leprosy bacillus, it has thus far defied all efforts at cultivation *in vitro*, whether in bacteriological media or in cell cultures (3). Zinsser and Carey were the first to report the intracellular multiplication of this organism in spleen explants *in vitro* (4). Evaluation of this claim as well as of subsequent ones by other investigators is difficult. Though previous studies in this laboratory[†] have demonstrated high infectiousness in *M. lepraemurium* maintained in rat fibrocytes for 30 days, there were no significant increases in bacterial numbers. The problems involved in making microscopic determinations in cellular systems containing mycobacteria are considered in detail elsewhere (5). Metabolic studies (6,7,8) have elucidated many of the peculiarities of *M. lepraemurium*; especially the disruption of its endogenous metabolism and infectiousness by the serum concentrations previously employed for growing tissue cells *in vitro*. *In vivo* experiments have shown that during logarithmic growth, when bacterial numbers double in 8-10 days, bacilli are being destroyed in some infected cells while flourishing in others (9). This observation is possibly explained by the experimental finding that only 15-20% of freshly harvested bacilli are metabolically active.[‡]

The present paper reports a 2-3 fold increase of *M. lepraemurium* in cell culture systems in which the following circumstances prevailed: 1) Cells containing a high incidence of active microorganisms were assured by transplanting spleen cells from mice at a time when

the bacilli were in the logarithmic phase of growth. 2) For *in vitro* infection by means of bacillary suspensions, each phagocytic cell was permitted to acquire on the average some 10 bacilli which had been protected in serum albumin and yeast supplement (6). 3) The probable impediment imposed by inhibitors in serum was minimized by nurturing the cells in fluids containing low concentrations of body fluids. All supplements were known to exert no deleterious effects on the metabolism of the bacilli. 4) Bacillary numbers were determined before and after incubation by combining the microorganisms from culture supernates with those liberated from disrupted cells and making direct microscopic counts.

Materials and methods. *M. lepraemurium*: The Wells strain was used in cells of infected mouse spleens without prior storage. Suspensions of the Hawaiian strain of *M. lepraemurium* were prepared from infected rat testes. Following light centrifugation, these bacilli in 20% tissue homogenates were refrigerated in 5% albumin and 5% Difco yeast supplement B. *Tissue culture.* Spleen tissue of mice infected with the Wells strain of *M. lepraemurium* for 3-6 weeks *in vivo* was minced and "Mag-mixed" in pancreatin 0.25% for 30 minutes. Suspended cells were decanted from undigested tissue, spun lightly to remove enzyme and extracellular organisms, and resuspended in a medium composed of mouse serum 10%, human ascitic fluid 10%, Eagle's amino acids, vitamins, and glutamine (10), chick embryo extract 1%, and BSS. Aliquots were frozen for control bacillary counts and distributed to tubes for incubation at 34°C. Stock cells of clone 929, Earle's L strain of mouse fibrocytes,[‡] were maintained in: horse

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† J. H. Hanks—unpublished observation.

‡ We are indebted to Dr. Charity Waymouth for this sub-line adapted to growth in the absence of serum or body fluids; for the trace mineral solution; and for many helpful suggestions.

serum 2%, Eagle's mixture, Bacto-peptone 0.5% (11), Waymouth's trace minerals \S , M/560 Na_2HPO_4 , and Hanks' BSS. These cells were infected by combining equal amounts of a 0.5% tissue suspension of the Hawaiian strain of *M. lepraemurium* in medium with a pancreatinized suspension of cells ($10^6/\text{ml}$) from stock cultures. Aliquots of this mixture (0.2 ml) were distributed to 15 mm tubes in which 12 mm round cover slips had been seated in 0.3 ml of medium. After incubation at 34°C for 24-48 hours, cells were pancreatinized from the coverslips, centrifuged lightly to separate intact infected cells from damaged cells and extracellular bacilli, and again pooled. Aliquots containing 100,000 infected cells were distributed to fresh cover slips and incubated at 34°C . The day of replanting was regarded as day zero. Aliquots also were frozen for subsequent control observations and bacillary counts. *Bacillary counts.* In early experiments, cells were pancreatinized from 1-3 cultures, pooled with supernatant fluids, and centrifuged at $3300 \times G$ for 30 minutes. Supernatant fluids were removed to 0.1 ml per sample and sediments were frozen and thawed 3 times and aspirated vigorously through a small needle to encourage cell rupture. 0.001 ml of each resulting suspension was spread on five 5×5 mm areas on microscope slides by means of an Agla micrometer syringe. Films were heat fixed, Ziehl-Neelsen stained, and differentiated in sulfuric 4%-methylene blue (12). Bacilli were enumerated in 20 randomly chosen oil immersion fields in 2 to 5 films/sample. The differences between counts were analyzed by the paired replicate method of Wilcoxon (13). In later experiments, analyses were made of the counts recorded in each of 15 fields across the diameters of microspots delivered to slides by means of a standard 0.7 mm platinum loop. This method has been described and analyzed by Hanks (5). The homogeneity and reproducibility of samples was improved as follows: Bile 0.05% was incorporated in the pooled samples in 10 mm lusteroid tubes prior to high speed centrifugation. After the volumes of concentrates had been adjusted to 0.1 ml per sample, these tubes were packed

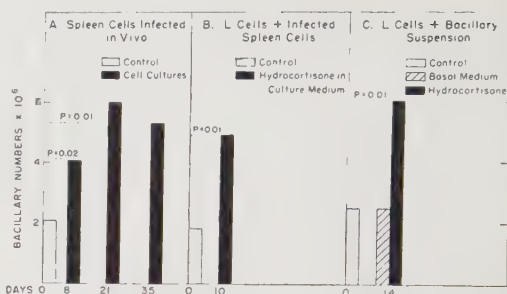


FIG. 1. Multiplication of *M. lepraemurium* during incubation in cell cultures.

in a 10 KC Raytheon ultrasonicator (water depth around tubes = 15 mm) and oscillated at 150 micro-amperes for 2 minutes. The incorporation of bile is necessary to prevent abnormal distributions of bacilli during the drying of such homogenates on films. Reliable fixation necessitated exposure of flame-fixed slides to formalin fumes for 8 minutes prior to staining and differentiation.

Results. Multiplication of *M. lepraemurium* in cells *in vitro* has been initiated under 3 circumstances of cellular infection and within 2 cell types.

The first type of experiment consisted of direct explantation of spleen cells from mice infected with the Wells strain of *M. lepraemurium*. Results shown in panel A, Fig. 1, represent initial counts compared with those made after incubation for 8, 21, and 35 days. Since bacillary numbers nearly doubled within 8 days, proliferation during this period occurred at the maximal rates observed during infection of susceptible hosts. After 21 days, the counts were $2.8 \times$ the original. After 35 days the counts were beginning to decline, possibly because of deterioration of the cell populations between the 21st and 35th days. Rees and Wong at the National Institute for Medical Research, London, have obtained similar results with spleen explants from *M. lepraemurium* infected mice. \S

An interesting modification of the foregoing experiment consisted in ascertaining whether infected mouse cells could be employed as a means of transferring *M. lepraemurium* to an established line of fibroblastic cells. Preliminary studies showed that during incubation for 48 hours in low serum medium appreciable numbers of the inadequately

\S Personal communication.

maintained mouse spleen cells were phagocytized by L cells. In the conduct of such experiments the *in vitro* infected L cells were pancreatinized after 48 hours, pooled, and redistributed to new coverslips. At this time aliquots were also stained for observations of original cell-bacillary relationships, and other aliquots were frozen for subsequent control bacillary counts. Microscopic study of incubated cultures demonstrated that some of the L cells were successfully parasitized while others obviously had destroyed mycobacteria. Counts in such systems demonstrated no bacillary increases despite the fact that the medium contained a low concentration of serum and the bacilli had not been exposed to extracellular environment. Apparent bacillary success in some cells was offset by the resistance and/or high metabolic capacity of others.

Grossfeld has described modifications of cell metabolism by hydrocortisone and has shown that the compound permits cultured cells to survive without feeding for significantly longer periods than controls(14). The above experiment in which spleen cells were combined with L cells was repeated. However, after pancreatinization and pooling of infected L cells, aliquots were replanted in low serum medium containing hydrocortisone|| at the level of 0.2 mg/ml. As shown in Panel B, Fig. 1, after 10 days incubation the numbers of bacilli in the modified L cells had increased to approximately 2.5 times the initial numbers. Further incubation did not result in significant increase of *M. lepraemurium*.

In the previous experiments, bacilli were protected from extracellular environment during transfer to *in vitro* systems. Trials were then made by adding to L cells the Hawaiian strain of *M. lepraemurium* which had been suspended and refrigerated in albumin and yeast supplement. These organisms maintained their microscopic hydrogen transfer capacity during their acquisition by the L cells.¶

|| Through the courtesy of Dr. J. C. Richards, Merck Sharp & Dohme Research Laboratories, Division of Merck & Co.

¶ Unpublished data.

Hydrocortisone (0.2 mg/ml) was incorporated in the medium used for replanting a portion of the infected cells. Panel C, Fig. 1, representing the average of several determinations, shows that the bacillary increase obtained by this procedure resembled that already described. After 14 days incubation the increase of bacterial numbers was 2.7 times the original counts. Again prolonged incubation resulted in no further increase. The counts did not rise when hydrocortisone was omitted from the medium.

Because of the limited increases in bacillary numbers in the foregoing experiments, attempts were made to demonstrate: (a) that the more prolonged freezing of bacilli in control samples might permit attrition by ice crystals or make the organisms more susceptible to sonic disruption, and (b) that breakage of the longer bacillary elements into shorter lengths during their residence within cells might cause an apparent rather than a true rise in counts. A series of critical studies showed that bacillary numbers remained constant during freezing and thawing and during freezing for periods longer than those reported; and also that decreases in bacterial lengths played no part in elevation of the counts.

The present demonstration of limited multiplication of *M. lepraemurium* in cells *in vitro* probably has been made possible by adherence to the considerations outlined in the introduction. A definition of the exact role of these and other factors must await the development of cellular systems which are more favorable to continuous cycles of cellular infection.

Summary. Quantitative determinations have revealed significant multiplication of *M. lepraemurium* in explanted spleen cells which had been infected *in vivo*; also in the L strain of mouse fibrocytes infected *in vitro*, provided the metabolism of the latter was altered by means of hydrocortisone. During periods of 8-10 days, the rate of multiplication closely approximated the maximal rates observed in susceptible animals.

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Relationships between Bacterial Resistance to Serum and Penicillin. (23658)

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Despite numerous studies on differential resistance of bacterial species and strains to bactericidal effects of normal sera, only little information is available on the genetic aspects of bacterial sensitivity or resistance to serum factors(1-3). Where such information has been collected, antigenic alterations of the bacteria usually accompanied alterations in "serum resistance." Recently, there has been a resurgence of interest in bactericidal systems, due to recognition of the role of properdin(4-5) in such phenomena. Therefore, a renewed effort has been made to study certain genetic aspects of bacterial resistance with particular emphasis on closely related strains of similar antigenicity. In the course of these studies, changes in serum resistance without qualitative antigenic alterations and an inverse correlation between resistance to penicillin and to bactericidal factors of normal human serum were observed. These observations form the subject of the present report.

Methods. Smooth cultures of *Shigella dysenteriae* #377, and *Escherichia coli* SPI, sensitive to the properdin system(4), were obtained from Dr. R. J. Wedgwood. These cultures proved extremely heterogeneous on the basis of colonial morphology when inspected by oblique lighting(6). Single colony isolates were used to establish substrains with greater colonial homogeneity, displaying identical antigenic* and fermentation character-

istics. The susceptibility of these strains to normal human serum, obtained from a single donor, was determined by exposing a known number of bacteria (approx. 5000) for various periods of time at 37°C to serum diluted with ABA buffer (Michaelis buffer, pH 7.5, containing albumin(4)). The concentration of serum in buffer varied from 1:6 to 1:2.5 depending upon the sensitivity of the strain tested, but the total volume during exposure time was always 1.2 ml. Bacteria for testing were harvested from 18-hour tryptose agar slants and plated on tryptose agar (in 0.1 ml aliquots) following exposure to serum. Prior to plating and prior to incubation at 37°C the bacterial suspensions in serum were kept in an ice-bath. Sera were stored at -40°C. All tests were done at least in duplicate and duplicate samples of replicate cultures were assayed.

Results. Six colonial types were recognized when the original culture of *Sh. dysenteriae* was inspected on McConkey agar. Four of these yielded stable subcultures differing in their resistance to the bactericidal effects of normal human serum; the other 2 isolates proved to be unstable in regard to colonial morphology and antigenic characteristics and were not studied further. Table I demonstrates typical differences in serum resistance between the most sensitive (S_3) and most resistant (S_1) of the stable substrains isolated from the original heterogeneous culture.

When attempts were made to convert mem-

* Based on reactions with specific antiserum and acriflavine.

TABLE I. Resistance of Closely Related Strains of *Sh. dysenteriae* to Normal Human Serum (Serum Sample #H2).

| Strain | Serum conc. | % survival after | | | ET ₅₀ (10) | Sensitivity to penicillin (units/ml) |
|------------------------------------|-------------|------------------|----------|-----------|-----------------------|--------------------------------------|
| | | 15' | 30' | 60' | | |
| S ₁ | 1:6 | 94 ± 5.2 | 92 ± 5.4 | 76 ± 4.2 | 162' | 12.5 |
| S ₃ | " | 95 ± 3.5 | 24 ± 3.7 | 3.6 ± .5 | 18' | 25 |
| S ₁ -pr ₁₂ * | " | 47 ± 6.5 | 22 ± 3.4 | 2.8 ± 2.0 | 12' | 700 |
| S ₁ | 1:2.5 | 67 ± 4.3 | 45 ± 7.1 | 28 ± 1.5 | 33' | 12.5 |
| S ₁ -pr ₅ | " | 41 ± 5.5 | 22 ± 2.2 | 13 ± 1.8 | 13' | 100 |
| S ₁ -pr ₈ | " | 34 ± 6.8 | 13 ± 2.2 | 5.3 ± 1.3 | 10' | 200 |

* Mutant isolated after 12 transfers of S₁ on gradient plates containing penicillin.

bers of these two representative strains into protoplasts by exposure to penicillin + sucrose(7), it was noted in the sucrose-free penicillin-containing (2000 units/ml) control suspensions that S₁ cells were lysed far more rapidly than S₃ cells. Subsequent measurements of penicillin resistance of the strains differing in serum resistance revealed that increased resistance to one was associated with decreased resistance to the other (Table I).

To verify the generality of this relationship, mutants with increased resistance to penicillin then were isolated from the sensitive S₁ strain by transfers on gradient plates(6). The serum resistance of the recovered mutants, showing stepwise increases in penicillin resistance, was assayed following one or more transfers on penicillin-free media and, as illustrated in the lower part of Table I, confirmed the inverse correlation between the 2 resistance characteristics. Resistance to normal serum factors decreased as resistance to penicillin increased. Similar observations were made with *E. coli* strains.

Penicillin is known to interfere with the synthesis of bacterial cell-wall components (8). Therefore, it could be expected that compensating alterations in the cell-wall of

resistant mutants are responsible for the altered sensitivity to bactericidins. To provide a basis for further elucidation of such relationships, the serum resistance of genetically identical cells was compared following 18 hours of pregrowth in plain broth or in broth containing noninhibitory concentrations of penicillin. As shown in Table II, prior exposure to penicillin did produce significant phenotypic changes in serum resistance; bacteria pregrown in sufficiently low concentrations of penicillin proved significantly more resistant to serum than their sister cells grown in plain media. Possible causes for such effects will be discussed below. An interesting zonal effect also was observed: pregrowth in 0.5 or 5 units of penicillin per ml protected against subsequent serum effects, pregrowth in 1 or 2 units/ml did not (the actual units differ depending upon the strain employed). Also, pregrowth in penicillin concentrations that are close to the inhibitory level (e.g. S₁ in 10 units) have effects opposite to those just described; they render the cells more sensitive to subsequent serum treatment. This can be explained on the basis of piling one injurious effect upon another, similar to the synergistic effects of other antibiotics and serum noted by others(2) during

TABLE II. Effect of Pregrowth in Penicillin-Containing Media upon Subsequent Susceptibility of *Sh. dysenteriae* Cells to Normal Human Serum (Serum Sample #H3).

| Strain | Penicillin in pregrowth medium (units/ml) | Serum conc. | % survival after | | | ET ₅₀ | Sensitivity to penicillin (units/ml) |
|----------------------------------|---|-------------|------------------|----------|-----------|------------------|--------------------------------------|
| | | | 30' | 60' | 90' | | |
| S ₁ | | 1:2.5 | 54 ± 6.2 | 23 ± 3.1 | 3.9 ± 1.1 | 32' | 12.5 |
| " | 5 | " | 75 ± 3.9 | 63 ± 4.1 | 48 ± 2.3 | 84' | 12.5 |
| S ₁ -pr ₁₂ | | 1:6 | 33 ± 3.4 | 12 ± 1.6 | 3.9 ± .6 | 19' | 700 |
| " | 100 | " | 67 ± 4.2 | 40 ± 3.6 | 21 ± 2.8 | 42' | 700 |

simultaneous exposures. In additional studies with *Sh. dysenteriae* strains from other sources (Walter Reed Army Hospital), it was observed that occasionally differences in serum resistance occur that are not correlated in a simple fashion[†] with differences in sensitivity to penicillin. This may indicate that there are at least two different mechanisms leading to altered bacterial resistance to serum. Finally, it has been determined that the presence of 20% sucrose, but not of 20% lactose, prevents all bactericidal effects of serum. The nature of this protection is now being studied, and it remains to be seen whether or not it is related to the carbohydrate effects previously observed by Maaløe (1).

Discussion. The foregoing observations demonstrate that considerable heterogeneity in regard to resistance to bactericidal effects of normal human serum can exist in cultures believed to be relatively homogeneous on the basis of antigenic characteristics. Differences in serum resistance that occur independently of detectable antigenic changes have been noted before (1), but the recognition and control of such genetic alterations in stock-cultures of bacteria used for the testing of bactericidal systems has been generally neglected. In the case of *Sh. dysenteriae* and *E. coli*, cultural heterogeneity in regard to serum resistance now has been shown to be detectable by checking colonial morphology with the aid of oblique lighting on McConkey agar. Various other media have been tested but failed to produce comparable differentiation. Nevertheless, it is probable that entirely different media may be required to detect such differences in still other species.

More data are required to elucidate the actual nature of the alteration in penicillin-resistant cells that renders such cells more sensitive to serum and is modifiable by pregrowth in noninhibitory concentrations of the antibiotic. The alteration is presumably in the cell-wall, since penicillin is known to interfere with cell-wall synthesis (8), and since the cell

wall represents the initial locus of reaction for serum bactericidins. It is generally assumed that each cell may possess several reaction sites for serum factors, that a minimum number of these must be affected for cidal effects, and that the bactericidins distribute themselves among the exposed cells according to the binomial distribution. Therefore, the change occurring with increased resistance to penicillin may either effect 1) a change in total number of reactive sites per cell, 2) chemical reactivity of these sites, or 3) a change in the minimum number of reactions per cell required for killing. Differences in the killing end-points, obtained by extrapolating the present data to infinite time, appear too great to support the simplest assumption, namely, that penicillin resistance and serum resistance have been altered merely by a quantitative change in cell-wall material and reactive sites. The possible involvement of qualitative changes may become apparent as additional titration data become available.

Finally, the observation of an inverse correlation between resistance to penicillin and resistance to serum may help to explain the known occurrence of two general groups of penicillin-resistant pathogens, detected primarily in studies with staphylococci (9). It will be recalled that resistant organisms isolated from clinical cases as a rule owe their resistance to the production of penicillinase, whereas resistant mutants isolated *in vitro* fail to produce penicillinase and generally are of decreased virulence. The low survival value *in vivo* of the latter group of mutants now may be attributed to their increased sensitivity to normal serum bactericidins. In contrast, mutants resistant to penicillin by virtue of penicillinase production can be assumed to possess unaltered resistance to serum and thus a higher survival value *in vivo*.

Summary. Substrains isolated on the basis of colonial morphology from antigenically homogeneous cultures of *Sh. dysenteriae* and *E. coli* displayed stable genetic differences in resistance to the bactericidal effects of normal human serum. This serum resistance was inversely correlated with penicillin resistance: with increased resistance to the antibiotic there was an increased susceptibility to serum.

[†] There is some preliminary evidence for differences among these strains in rate of lysis at a given penicillin concentration.

Mechanisms that may be responsible for this relationship, and their bearing on certain problems of *in vivo* penicillin resistance, have been discussed.

The constructive interest of Dr. Otto Plescia is acknowledged with gratitude.

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Distribution of Terminal Temperatures in Hypothermic Dogs. (23659)

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Anesthetized dogs subjected to acute progressive hypothermia succumb in one of 3 ways(1). Under pentobarbital anesthesia the majority (60%) terminate in ventricular fibrillation (VF) at heart temperatures of 25°-19°C, terminus usually being preceded by extrasystolic activity. A proportion (15%) terminate in asystole at 18°-15°C. The remainder (25%) exhibit a period of asystole of variable duration (5-80 sec.) or an exceedingly slow heart rate (less than 16 b.p.m.) either of which is followed by VF. The latter type of death has been termed "asystolic fibrillation" (AF) and occurs at temperature range intermediate between the 2 former groups, *i.e.* 20-17°C(1).

It would be of interest to discover whether each group represented a distinct segment of the population or whether each type of death was a direct consequence of the terminal temperatures which were otherwise distributed along a predictable curve. Statistically the question is whether the population of animals employed was homogeneous regarding susceptibility to VF, *i.e.* whether terminal temperatures are uniformly distributed as opposed to a distribution exhibiting bi- or trimodalities. This could be determined only after a large group of "control" experiments had accumulated. Such a series of animals, simi-

larly treated, is now available to make the analysis possible.

Methods. Results from a total of 110 mongrel dogs of both sexes, weighing between 8-18 kg, are included. All animals were anesthetized with pentobarbital (30-35 mg/kg) and cooled to terminus by immersion in an iced bath. Details of the method have been previously reported(2). Heart temperatures were measured thermoelectrically with a catheter thermocouple inserted into the right auricle via the right jugular vein. Rectal temperatures were measured with a rectal thermocouple. The accuracy of the temperature recording instrument is within $\pm 0.5^\circ\text{C}$. All animals were allowed to breathe spontaneously until a heart temperature of $25 \pm 1^\circ\text{C}$ was reached. At lower temperatures, they were respired artificially by means of a positive pressure respirator.

Results. When all terminal heart temperatures were plotted in a frequency nomogram, it became apparent that they deviated greatly from a Gaussian distribution. Nevertheless there was no evidence of bimodality to suggest that the animals terminating in asystole or asystolic fibrillation were derived from a different population from that showing extrasystoles followed by VF.

A simple transformation of the terminal

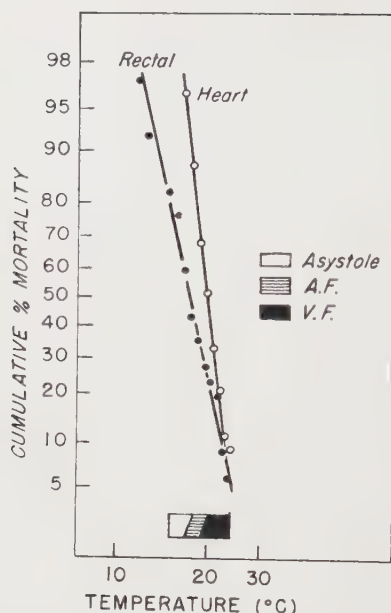


FIG. 1. Log-normal distribution of terminal temperatures in hypothermic dogs.

temperatures into their logarithmic equivalents revealed that this distribution, like many other biological variables, was in fact a log-normal distribution(3); *i.e.* the logarithms of the terminal temperatures were distributed along a normal Gaussian curve. Thus a probit-log temperature plot according to the method suggested by Gaddum(3) gives a good approximation of a straight line (Fig. 1) which is verified by a chi-square test using the method of maximum likelihood(4).

Similar results were obtained from the analysis of the rectal temperatures although the slope of the line and the median are different. This would be expected since it is known that in moderate hypothermia rectal temperatures are usually lower than heart temperatures and the 2 measurements show no strict parallelism during further cooling (2).

The median lethal temperature (LT_{50}) was found to be 20.1°C for heart temperatures and 19.8°C for rectal temperatures. The 95% confidence limits respectively were: 21.3 to 18.9 and 23.8 to 17.8.

Discussion. The analysis indicates that under the experimental conditions employed, the terminal temperatures of hypothermic

dogs are distributed uniformly along a log-normal distribution curve with no evidence that the population of dogs was heterogeneous regarding susceptibility to hypothermia. It appears rather that dogs terminating in asystole or AF do not belong to a different segment of the population from those succumbing to VF. It seems most probable, therefore, that terminal events are related to terminal temperatures rather than vice versa. This is of particular importance, since it places major emphasis on terminal temperature rather than mode of death.

However, this conclusion may not be valid in the case of animals subjected to modifications of the standard procedure, *e.g.* administration of pharmacologic agents, since such agents may depress the myocardium and precipitate asystole at relatively high temperatures, or conversely predispose to fibrillation at temperatures above or below the range of controls. Nevertheless, the analysis here presented provides a potent tool for the investigation of pharmacologic or other agents on the responses of the hypothermic heart. Probit-log temperature curves can be plotted for treated and control groups and a statistical comparison made which would include not only the median temperature and its confidence limits but also the slope of the probit line, *i.e.* the spread of the mortality distribution.

Summary. The distribution of terminal temperatures following induced hypothermia in dogs was statistically analyzed for evidence of inhomogeneity. It was found that the data from over 100 animals could be adequately expressed by a log-normal distribution without any evidence of heterogeneity. This suggests that the different terminal events (*i.e.* fibrillation *vs.* asystole) which occur under induced hypothermia are determined by the terminal temperatures rather than vice versa.

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Creatine Metabolism in Vitamin E Deficiency.* (23660)

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It is well established that creatine metabolism is deranged in vit. E-deficient animals. The general syndrome is characterized by an increased concentration of liver creatine, an increased urinary excretion of creatine and by a decreased concentration of creatine in skeletal muscle. This condition may be the result of an impaired incorporation of creatine into skeletal muscle of vit. E-deficient animals, thus leading to an increased creatinuria, or alternatively creatine may be poorly retained in the muscle, thus resulting in a rapid turnover of skeletal muscle creatine and its subsequent excretion in the urine. Data submitted in previous reports have supported the latter alternative(1,2). The present experiments were designed to measure directly the influence of Vit. E deficiency on the turnover rate of liver creatine and muscle creatine in rabbits.

Methods. The preparation of diets, Vit. E supplementation and general handling of the rabbits was the same as previously described (3). When the rabbits receiving the unsupplemented diet developed the usual deficiency signs they were selected for experiment. Four normal and 4 Vit. E-deficient rabbits were each injected intraperitoneally with 100 μ c of glycine-1-C¹⁴ (specific activity 0.58 mc per mM) per kilo of body weight. One normal and one deficient animal were killed 1.5, 3, 4, and 6 hours after the injections. The concentrations and specific activities of kidney glycocyamine, liver creatine, skeletal muscle creatine, and heart creatine were determined. Approximately 10 g of kidney were homogenized with an equal volume of water. Trichloroacetic acid (TCA) was added to a final concentration of 5% and the protein was removed by centrifugation. One milliliter of the supernatant solution was taken for glycocyamine determination by the permutit

procedure(4). Carrier glycocyamine (100 mg) was added to 10 ml of the TCA supernatant solution and dissolved by warming. The pH was adjusted to 7 and the glycocyamine was allowed to crystallize in the ice box. The glycocyamine was recrystallized from water to constant radioactivity as assayed with an end window Geiger tube. The counts were corrected to infinite thinness. The specific activity of the glycocyamine was then multiplied by the total amount of carrier added to give the total glycocyamine counts in the 10 ml of TCA supernatant solution. Based on the glycocyamine concentration of the TCA supernatant solution before carrier addition, as determined on the separate one ml aliquot, it was possible to calculate the specific activity of the kidney glycocyamine. Muscle and liver creatine specific activities were determined after conversion to creatinine. Three grams of muscle or 10 g of liver were cut in small pieces, placed in a large test tube containing 20 ml of 2 N H₂SO₄ and heated for 3 hours in a boiling water bath. After cooling, 18 ml of 2 N NaOH and 5 ml of 10% sodium tungstate were added to the tubes. The contents were filtered into a heavy duty test tube containing approximately 200 mg of Lloyd's reagent. The filtrate and Lloyd's reagent were mixed by repeated inversion for 10 minutes and then centrifuged. The creatinine formed during the heating of the tissue in acid was now adsorbed on the Lloyd's reagent. This was transferred to a 12 ml conical centrifuge tube, washed 3 times with 2 ml portions of 0.01 N HCl and centrifuged. To the sediment were added 4 ml of 0.5 N NaOH, the contents of the tube were mixed by repeated inversion for 10 minutes and centrifuged. The sediment was re-extracted with 2 ml of 0.5 N NaOH and the supernatant fluids were combined. This fraction contained the creatinine eluted from the Lloyd's reagent and was used for quantitative determination of creatinine and for the isolation of creatinine for counting. Creatinine was deter-

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† Studies carried out during tenure of Lederle Medical Faculty Award.

TABLE I. Incorporation of Glycine-1-C¹⁴ into Glycocyamine and Creatine by Normal and Vit. E-deficient Rabbits.

| Measurements | Hr after inj. | Conc. (μ M/g) | | Specific activity (c.p.m./ μ M) | |
|--------------------------|---------------|--------------------|-------------|-------------------------------------|-------------|
| | | Normal | E-deficient | Normal | E-deficient |
| Kidney glycocyamine | 1½ | .42 | .42 | 1050 | 408 |
| | 3 | .64 | .57 | 31 | 170 |
| | 4 | .43 | .39 | 390 | 171 |
| | 6 | .42 | .34 | 206 | 115 |
| Liver creatine | 1½ | .30 | 1.21 | 424 | 206 |
| | 3 | .18 | .59 | 19 | 142 |
| | 4 | .28 | 1.05 | 313 | 95 |
| | 6 | .15 | 1.69 | 303 | 41 |
| Skeletal muscle creatine | 1½ | 37 | 23 | .9 | 3.9 |
| | 3 | 42 | 24 | .1 | 4.7 |
| | 4 | 32 | 8 | 2.8 | 10.2 |
| | 6 | 37 | 14 | 3.9 | 3.7 |
| Heart creatine | 1½ | 13 | 15 | 17.1 | 19.6 |
| | 3 | 11 | 12 | 1.8 | 14.0 |
| | 4 | 22 | 10 | 23.0 | 4.5 |
| | 6 | 12 | 21 | 21.9 | 2.5 |

mined by a micro procedure, in which 0.025 ml of the NaOH extract was diluted with 4 ml of water in a Klett colorimeter tube. Two milliliters of freshly prepared alkaline picrate (one part 10% NaOH and 5 parts saturated picric acid) were added and after 20 minutes the tubes were read in a Klett photoelectric colorimeter with a green filter. The creatinine concentration was calculated by comparison with a standard curve. To the remainder of the NaOH extract, after neutralization with 5 N HCL, were added 100 mg of creatinine. After the creatinine was dissolved 4 ml of 95% ethanol and 2 ml of 20% zinc chloride were added. The tubes were placed in the ice box overnight and the precipitated creatinine zinc chloride was recrystallized to constant radioactivity. The specific activity of the original creatine was calculated as described for kidney glycocyamine.

Results. The data are given in Table I. It should be noted that Vit. E deficiency resulted in an increased concentration of liver creatine, a decreased concentration of skeletal muscle creatine, and was without effect on the concentration of kidney glycocyamine. These findings are in agreement with other reports(5). Also the concentration of heart creatine was not significantly affected by Vit. E deficiency.

It is well established that in mammals glycocyamine is synthesized in the kidney and methylated to creatine in the liver. This liver creatine may then be incorporated into skeletal muscle or cardiac muscle. In order to estimate the turnover rate of liver creatine, the specific activity of liver creatine was divided by the specific activity of kidney glycocyamine from the same animal. This factor was then multiplied by the concentration of liver creatine expressed in μ moles per gram. The value so obtained was considered to represent the μ moles of creatine per g of liver which had been replaced in the time interval between injection of the radio glycine and killing of the animal. Similarly, to estimate turnover rate of muscle creatine, the specific activity of muscle creatine was divided by the specific activity of liver creatine from the same animal and the result multiplied by the concentration of muscle creatine.

Fig. 1 presents the results of calculation of the turnover rate of liver creatine. It is apparent that in the E-deficient animals all the liver creatine had been renewed within 1.5 hours. In contrast, normal animals required approximately 4 hours to renew all of their creatine. These results show clearly that the rate of creatine synthesis is elevated in Vit. E-deficient rabbits. Such a conclusion has been reached by other workers(6).

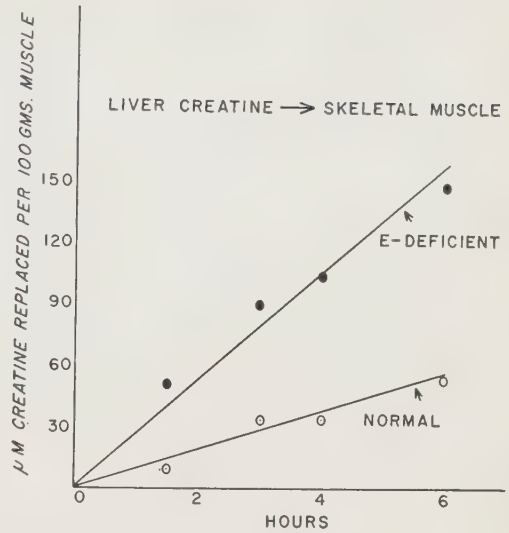
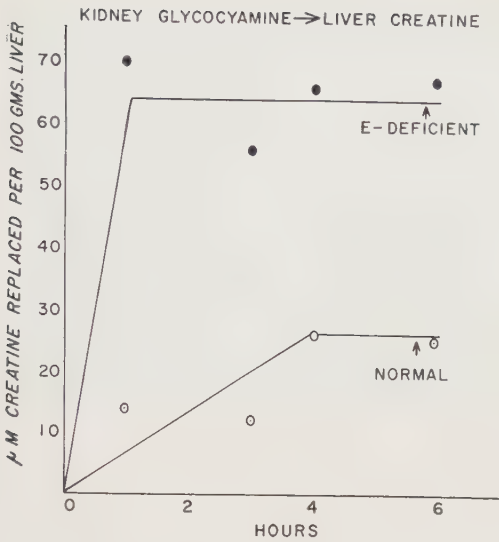


FIG. 1 (left). Creatine turnover in livers of normal and vit. E-deficient rabbits.

FIG. 2 (right). Creatine turnover in skeletal muscle of normal and vit. E-deficient rabbits.

The turnover rate of creatine in skeletal muscle is illustrated in Fig. 2. It is quite apparent that in Vit. E-deficient rabbits the turnover rate of muscle creatine is increased. This conclusion is in agreement with our earlier observation(1,2).

The turnover rate of heart creatine was also calculated and it was found to be unaffected by Vit. E deficiency. It should be mentioned however that heart creatine was found to be renewed at a considerably greater rate than the creatine of skeletal muscle.

The results of these experiments indicate that the creatinuria of Vitamin E deficiency is due, at least in large part, to an inability of the skeletal muscle to retain creatine after its incorporation. In preliminary experiments, it has been found that when normal and Vit. E-deficient rabbits are injected with inorganic P^{32} there is no depression of the incorporation of the P^{32} into phosphocreatine in the deficient animals. This strongly suggests that the inability of the dystrophic muscle to retain creatine is not due to an impaired phosphorylation. The explanation for this defect will probably require more basic information on the state of creatine in muscle and on the factors which influence its stability.

Summary. 1) Normal and Vit. E-deficient rabbits were killed at varying time intervals

after injection of glycine-1- C^{14} . Concentration and specific activities of kidney glyco-cyamine, liver creatine, skeletal muscle creatine and heart creatine were determined. Vit. E deficiency led to an elevated concentration of liver creatine, to reduced concentration of skeletal muscle creatine and did not influence the concentration of kidney glyco-cyamine or heart creatine. 2. The turnover rate of liver creatine and skeletal muscle creatine was increased in Vit. E-deficient rabbits. The turnover rate of heart creatine was considerably greater than the turnover rate of skeletal muscle creatine and was unaffected by Vit. E deficiency. The results show that in Vit. E deficient rabbits, rate of creatine synthesis is increased and turnover rate of skeletal muscle creatine is increased.

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Production by Semicarbazide of Gross Skeletal Changes in Rats Similar to Osteolathyrism.*† (23661)

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Neuman, Maxwell and McCoy, in an attempt to produce liver lesions in chick embryos similar to those produced by the chemically related aminoguanidine, injected solutions of semicarbazide HCl into yolk sacs of incubating White Leghorn eggs(2). While semicarbazide had little or no effect on the developing chick liver, it produced skeletal changes which seemed to me to be strikingly similar to lesions which have been produced in the chick embryo by β -aminopropionitrile (BAPN)(3). Since BAPN produces osteolathyrism in the rat(4-6), it seemed likely that semicarbazide might have a similar effect.

Methods. Weanling, male rats of the Sprague-Dawley strain were used. The basal diet was Rockland Rat Stock Diet, finely milled by a commercial miller. Experimental diets were made up by drying solutions of semicarbazide HCl (SCH)† on suitable quantities of milled Rockland Diet. The diets were supplied *ad libitum*. All animals were autopsied at death or at the end of the experimental periods.

Results. In a preliminary experiment, rats weighing 60-62 g were divided into groups of 3 and fed diets containing 0.1%, 0.5%, and 1.0% SCH respectively. All rats receiving 0.5%, and 1.0% levels of SCH lost weight and died within 10 days. None of these exhibited any gross symptoms of lathyrism. The animals which received the 0.1% level of

SCH developed skeletal exostoses, spinal curvatures and thoracic cage deformities similar to those seen when BAPN or *Lathyrus odoratus* (sweet pea) seeds are fed to weanling rats(4-8). One of these showed severe slipping of the right distal femoral epiphysis within 10 days. All 3 animals survived until sacrificed after 10 weeks of feeding.

In a second experiment rats weighing 42-51 g were divided into groups of 10 and fed diets containing 0.05%, 0.1% and 0.25% SCH respectively. Growth rate was depressed in proportion to the level of SCH in the diet as shown in Fig. 1. On the lowest level growth was good, approaching that of rats fed unsupplemented Rockland stock diet. All groups developed skeletal lesions typical of osteolathyrism.

Symptomatology. The gross skeletal lesions

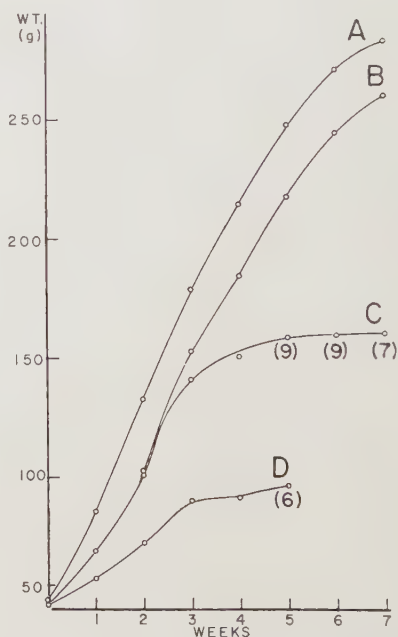


FIG. 1. Growth curves of rats receiving SCH. A—normal controls; B—0.05% SCH; C—0.1% SCH; D—0.25% SCH. All points represent average weight of 10 rats except as noted in parentheses.

* Osteolathyrism is a term introduced by Selye(1) differentiating the syndrome produced by β -aminopropionitrile, aminoacetonitrile, and certain species of *Lathyrus* (e.g., *odoratus*), in which marked skeletal lesions occur, from *neurolathyrism*, in which the symptoms are primarily neurologic and which results from the ingestion of certain other species of *Lathyrus* (e.g., *sativus*) or bis-(β -cyanoethyl)-amine.

† Supported in part by grant, National Institute of Arthritis and Metabolic Diseases, P.H.S. The author is also grateful to Mr. Ribhi Nasreddin for preparation of diets and care and feeding of animals.

‡ Fisher Certified Reagent.

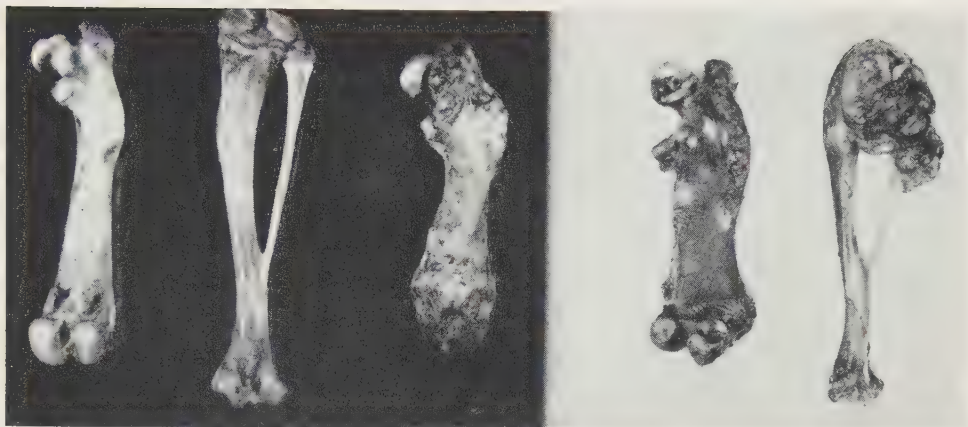


FIG. 2. Effect of SCH on femora and tibiae of weanling rats. Left: normal femur and tibia; center: 0.25% SCH for 28 days, distal articular surface damaged during removal; right: femur and tibia from rat fed 0.25% SCH for 56 days

were strikingly similar to those produced by BAPN(4-6). Exostoses on the ventral and lateral aspects of the mandibles could be palpated in rats on the 0.1% and 0.25% levels of SCH within 1-2 weeks after the feedings began. The long bones became thickened and prominent exostoses appeared at the sites of muscle attachment (Fig. 2). The bones of animals receiving the 0.25% level had the abnormal red color often seen in sweet pea lathyrism(9). Severe spinal curvatures and thoracic cage deformities occurred; even in rats receiving the lowest level of SCH (0.05%) marked spinal and thoracic cage deformities were present at the end of the experimental period (8 weeks). A marked S-curve at the base of the tail and more or less crooking or spiralling of the tail occurred in all rats, (Fig. 3). Crooking or spiralling of the tail has not been observed in rats receiving BAPN or aminoacetonitrile alone, but a marked curling of the tail has been reported by Selye in rats in which the effect of aminoacetonitrile was intensified by somatotrophic hormone(10). It should be stated, however, that the identification of the skeletal lesions with those produced by BAPN must await histological confirmation.

Neurologic symptoms occurred in all groups. These included paralysis of the rear legs, incontinence, paraphymosis, and atony of the colon. The incidence of neurologic manifestations increased with increasing levels of

SCH. Thus, the number of rats exhibiting paraphymosis at the 0.05%, 0.1%, and 0.25% levels of SCH were 2, 4, and 6, respectively (10 rats in each group). All these neurologic effects have been commonly observed in this laboratory in rats fed BAPN. As has been explained elsewhere in connection with a discussion of BAPN toxicity, it is felt that the neurologic symptoms are not due to a direct neurotoxic effect of the supplement, but are secondary to the osseous changes(11).

Dissecting aneurysms of the aorta, commonly seen in rats and mice fed BAPN or sweet peas(8,12-14) were not observed in any of the animals. One animal on the 0.25% level died of a ruptured saccular aneurysm of the aortic arch (non-dissecting) and one ani-



FIG. 3. Crooking of tails of rats fed SCH compared to normal at left. Rats photographed under nembutal anesthesia.

mal on the 0.1% level showed a grossly thickened and enlarged aortic arch (non-dissecting) when sacrificed at the termination of the experiment. The gross appearance of all other aortas was normal. Preliminary *microscopic* examination of the aortas, however, indicates that there was some damage to the aortic media in many of these animals.[§]

The incidence of deaths which occurred before the termination of the experiment (8 weeks) was as follows: 0.05% level, 0 of 10; 0.1% level, 3 of 10 (all from rupture of the urinary bladder due to severe urinary retention); 0.25% level, 7 of 8 (1 ruptured aorta, 1 ruptured urinary bladder, 5 cause unknown; 2 of original 10 rats were killed by mistake after 4 weeks by animal caretaker).

Discussion. Only relatively few chemicals have thus far been shown to be osteolathrogenic. The most prominent of these are β -aminopropionitrile (BAPN) (4-6) and aminoacetonitrile (6). The γ -glutamyl derivative of BAPN is responsible for the toxic properties of sweet peas (15) and of Singletary peas (16). Methyleneaminoacetonitrile has also been reported to be strongly lathrogenic (17). Bis-(β -cyanoethyl)-amine is mildly osteolathrogenic (4,6) but is markedly neurolathrogenic, *i.e.*, it produces central nervous system damage unrelated to the osseous lesions (4,18). Other substances closely related chemically to BAPN (4-6), even γ -aminobutyronitrile (6), the next higher homologue of BAPN, were found to be inactive. Only one nitrile has previously been reported to produce these skeletal changes. Commercially prepared β -mercaptoethylamine was found to cause similar skeletal lesions in our laboratory (5), but not in another laboratory (17).

The finding that semicarbazide produces lathritic skeletal lesions is important because it demonstrates again that the presence of a nitrile group is not a *sine qua non* for osteolathrogenic activity, since *in vivo* transformation of semicarbazide into nitriles is un-

likely. In addition, this observation opens up an entirely new group of substances among which lathrogenic action may be sought. It is still not clear whether osteolathyrism results from a stimulation of, or from an interference with, certain metabolic processes. It is hoped that the finding of new osteolathrogenic agents will aid in locating the metabolic defect of osteolathyrism.

Summary. Semicarbazide HCl, when fed to weanling male rats, produced gross skeletal lesions similar to those of osteolathyrism. Aortic damage seemed to develop more slowly and to be less severe than in rats treated with BAPN or aminoacetonitrile and having skeletal lesions of comparable severity.

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[§] With cooperation of Dr. R. V. Milliser, Dept. of Pathology.

Variations in Thyroid Morphology of Mice.* (23662)

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Variations in epithelial height and in the amount of stored colloid in the follicles have been associated with changes in the degree of activity of the thyroid gland. A high follicular epithelium and a small amount of stored colloid are characteristic of actively secreting glands, while a low epithelium and a large amount of colloid within the follicles are characteristic of less actively secreting glands(1). The rate of secretion of the thyroid hormones is controlled to a large extent by the production of thyroid stimulating hormone (TSH) by the pituitary, and observations of changes in thyroid morphology have been employed in various methods for TSH assay(2-4).

This investigation was initiated in order to determine if aging and parity might be associated with changes in thyroid morphology in 2 inbred strains of mice. The occurrence of spontaneous mammary tumors in some of the older C3H mice used in this study also enabled a comparison of the thyroids of these mice with those of tumor-free mice of corresponding ages.

Methods. Inbred Z(C3H)/Bittner/Lawrence and C57 Bl/6/JAX mice were used in this study. The mice were housed in transparent plastic cages and fed Purina Laboratory Chow *ad libitum* with an oat supplement. The temperature was rigidly controlled between 78-80°F without specific humidity control. Female littermates were randomized between the breeding and non-breeding study groups. Pregnant females were not isolated, but their litters were removed from the cage within 3 days after birth. However, about one month before sacrifice, most of the pregnant mice were isolated and were allowed to

nurse their last litter for 20-30 days, this last litter being weaned at least two days prior to sacrifice of the mother. Because of time considerations it was not possible to allow all of the animals of the 3 and 4 month groups to have such a period of lactation. Animals with tumors were isolated until they were sacrificed and those with ulcerated tumors were discarded. Animals were killed by cervical dislocation at monthly age intervals (± 5 days). They were sacrificed in several large batches, each of which included representatives from many of the groups. The trachea was used to handle the tissue and to enable consistent orientation of the thyroids for sectioning. The tissues were fixed in Bouin's fluid, dehydrated in alcohol, cut at 5 μ and stained with haematoxylin and eosin. The slides were coded to assure objectivity of the measurements. The mean of the measurements of 10 follicles in each of the 863 glands was recorded. The selection of the section to be measured was based on the clarity of the section as well as on the width of the thyroid lobe. Whenever possible, that section having the greatest width from its medial to its lateral aspect was selected. Ten representative follicles, which were midway between the anterior and posterior poles of the thyroid lobe and on a line extending from its medial to its lateral aspect, were measured.

Results. *Sex.* Analyses of measurements of both epithelial height and diameter of follicles revealed that there were no consistent differences between values obtained for males and for virgin females when animals of corresponding ages were compared. Therefore, the data from these 2 groups were pooled for all further analyses. This pooled sample of 652 mice hereafter will be referred to as the non-parous group.

Age. In both strains the non-parous mice showed a sharp decrease in thyroid epithelial height during the early months of life; from 1 through 3 months in the C3H strain and through 5 months in the C57, (Fig. 1). These

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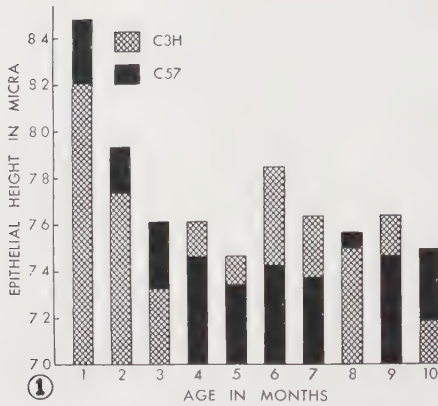


FIG. 1. Mean thyroid epithelial heights of non-parous C3H and C57 mice.

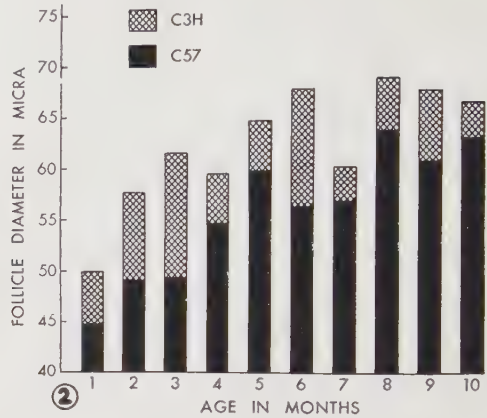


FIG. 2. Mean diameters of thyroid follicles of non-parous C3H and C57 mice.

differences are significant at the 1% level of confidence. Follicle diameter measurements from thyroids of non-parous mice exhibited an inverse relationship. That is, there was a marked increase in these values through the first 3 months in the C3H strain and through the first 5 months in the C57 strain, Fig. 2. In both strains there was a tendency for a continued, although less consistent increase in the follicle diameter size throughout the remainder of the 10 months of observation. A similar trend was also evident in the non-tumorous parous females, Fig. 3 & 4. These differences in follicular diameter measurements are also significant at the 1% level of confidence.

Parity. When data for epithelial height of tumor-free parous and non-parous C3H mice of corresponding ages were compared statistically, the observed differences were not significant, Fig. 5. However, in the C57 strain the thyroids of the parous females had a lower epithelial height than did those of the non-parous group at all ages except at the 5 month level, Fig. 6. Analysis of variance of the data in relation to parity indicates that the tendency for lower epithelial height in parous females in the latter strain is significant ($P = .01$). The differences in the mean follicular diameters of the parous versus the non-parous mice were not consistent, Fig. 3 & 4, but they did show a tendency for larger thyroid follicles to be present in the parous than in the non-parous mice ($P = .01$). The

exceptions were at the 3 and 6 month intervals in the C3H and at the 5 and 8 month intervals in the C57 strain. Measurements from 211 parous mice were employed for these analyses.

Strain. There was not a statistically significant difference in epithelial heights when C3H and C57 mice of corresponding ages were compared, Fig. 1. However, the mean follicular diameters of the C3H thyroids were significantly greater than were those of the C57 ($P = .01$), Fig. 2. The functional significance of these anatomical differences between the two strains is, however, questionable.

Presence of tumor. Data obtained from 21 tumor-bearing parous C3H mice 8-11 months of age were compared with those from 33 non-tumor parous C3H mice of the same age. The height of the follicular epithelium of the tumorous animals was found to be significantly lower ($P = .05$) than that of the non-tumor-

TABLE I. Thyroid Measurement of Tumor and Non-Tumor-Bearing C3H Mice.*

| Age in mo | Presence of tumor | No. mice | Mean diameter | Mean epithelial ht |
|-----------|-------------------|----------|---------------|--------------------|
| 8 | — | 8 | 70.35 | 7.69 |
| | + | 6 | 77.47 | 6.63 |
| 9 | — | 12 | 70.80 | 7.30 |
| | + | 9 | 79.05 | 6.74 |
| 10 | — | 12 | 74.50 | 7.39 |
| | + | 3 | 75.74 | 7.23 |
| 11 | — | 1 | 73.59 | 7.84 |
| | + | 3 | 78.54 | 7.44 |

* In micra.

ous animals. The follicular diameters were also correspondingly greater but these differences were not of statistical significance.

Discussion. Observations of a tendency for decreased epithelial height and increased diameter of thyroid follicles in both strains with increasing age, suggest that there is a decrease in thyroid activity with increasing age. These results are in agreement with other findings. Legait *et al.*(5) found increasing colloid accumulation in thyroids of rats as they matured. Hurst and Turner(6), using the assay method of Dempsey and Astwood, con-

cluded that mature albino mice of both sexes secrete less "thyroxine" than do younger mice. Grad and Hoffman(7) came to similar conclusions, employing rats.

The results of this study indicate that although there are no consistent differences between the thyroid morphology of males and of virgin females within either strain, the thyroids of the parous females of both strains show morphological evidence suggesting that they have a lower activity than do those in the non-parous group.

Tumor-bearing parous C3H mice also show

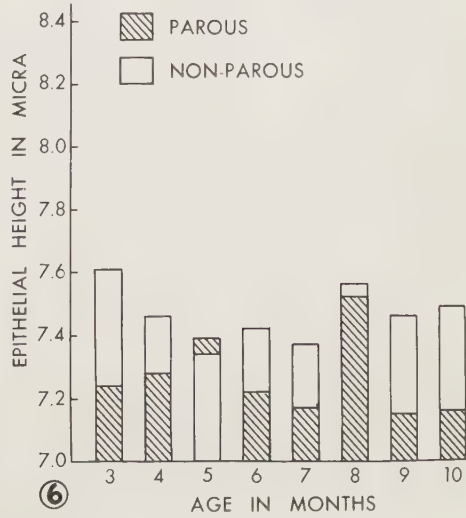
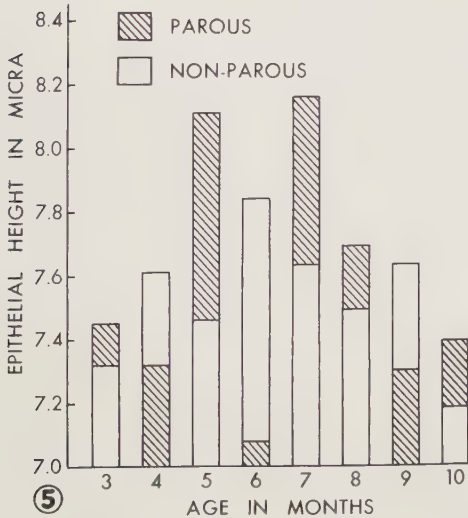
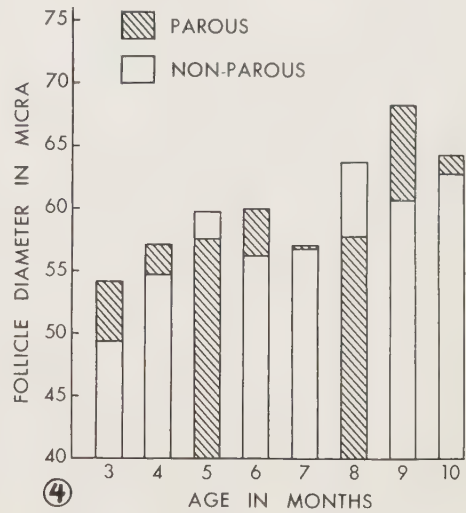
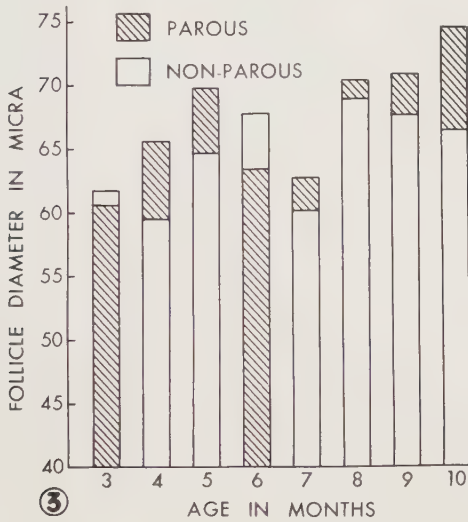


FIG. 3. Mean diameters of thyroid follicles of parous and non-parous C3H mice.

FIG. 4. Mean diameters of thyroid follicles of parous and non-parous C57 mice.

FIG. 5. Mean thyroid epithelial heights of parous and non-parous C3H mice.

FIG. 6. Mean thyroid epithelial heights of parous and non-parous C57 mice.

morphological evidence of decreased thyroid secretion when compared to tumor-free parous mice of corresponding ages. This is in agreement with the report of Larinow(8) that rabbits with tar-induced papillomata and mice with spontaneous and transplanted mammary adenocarcinoma show a depression in the height of the follicular epithelium. In these latter studies, the epithelium became increasingly lower as the tumors became larger. A lowered thyroid activity in tumor-bearing mice might well reflect metabolic changes within the host associated with the presence of tumor.

Summary. Observations of thyroid follicle epithelial heights and diameters were analyzed with respect to age and parity in C3H and C57 mice. Epithelial heights decreased and follicle diameters increased with aging and breeding. The presence of tumors in the C3H parous females was associated with de-

creased epithelial height and increased diameter of the thyroid follicles as compared to non-tumor-bearing mice of corresponding ages. These changes suggest that aging, breeding and the presence of tumor are associated with decreased thyroid function.

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Increase in Heidenhain Pouch Secretion after Portacaval Transposition in the Dog.* (23663)

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Several studies have indicated that peptic ulcers occur with greater frequency in patients with cirrhosis of the liver than in patients without cirrhosis(1,2,3). Malnutrition, deficient amounts of biliary elements, central nervous system damage, and inflammation and congestion of the stomach mucosa have been cited as possible causes(4). Baronofsky and Wangenstein(11) showed that partial obstruction to the venous outflow of the stomach in rabbits and dogs increases the speed and incidence of ulcer formation in response to injection of histamine in beeswax. In their animals a well-developed collateral circulation developed around the liver. Excess gas-

tric secretion of acid or pepsin has not been demonstrated in patients with cirrhosis(4), although studies on this point are few. Out of 29 patients who underwent portacaval shunt at the Veterans Administration Center, Los Angeles, since 1948 two, who had no previous ulcer history, developed peptic ulcers within 9 months after shunt. Four other cases have been reported of peptic ulcers which originated or underwent exacerbation after portacaval or splenorenal shunt(5,6). These observations throw some doubt on the importance of portal hypertension *per se* in the etiology of ulcers associated with cirrhosis. The presence of a well-developed collateral circulation through which portal blood bypasses the liver is a striking feature of many patients with cirrhosis and of all with func-

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tioning portacaval shunts. It occurred to us that gastric hypersecretion on a humoral basis might take place in the presence of significant shunting of portal blood around the liver, even though basal fasting secretion was normal or low. Suppose the secretory hormone from the gastric antrum (gastrin) were inactivated in the liver, and that under normal conditions only a fraction of the gastrin originally liberated from the antrum finally passed into the systemic venous circulation via the hepatic veins. Then if portal blood were shunted around the liver, an increased amount of gastrin might gain access to the systemic venous circulation and stimulate the gastric parietal cells to secrete acid. Such a phenomenon would not be detected by the usual tests of gastric secretion, but only by tests measuring the secretory response to antral stimulation.

To test this hypothesis the effect of portacaval transposition on acid secretion from Heidenhain pouches (no vagus innervation) was determined.

Methods. Heidenhain pouches were constructed in 4 dogs weighing between 12 and 24 kg. Each pouch was drained through an inlying stainless steel cannula into a rubber football bladder, allowing quantitative 24-hour collections of the pouch secretion as described by Dragstedt, *et al.*(7). The volume of each 24-hour collection was measured, and its free acid in milliequivalents was determined by titration with 0.1 N NaOH to the color change of Toepfer's reagent. The HCl output was calculated. Each dog was fed a constant amount (2, 3, or 4 cans) of a commercial dog food (trade name Thoro Fed) during the entire experiment. Water was allowed *ad lib*. The diet was supplemented daily with 1 g of NaCl for each 100 ml of pouch secretion. After 35 to 42 daily collections each dog underwent a portacaval transposition operation as described by Child(8). In this procedure all tributaries of the portal vein are divided from the junction of the superior mesenteric and splenic veins to its bifurcation at the hilum of the liver. The portal vein and inferior vena cava are transected, transposed, and the cut ends reanastomosed end to end. As a result the portal

TABLE I. Secretion from a Heidenhain Pouch before and after Portacaval Transposition.

| Dog | | No. of 24 hr col- lections | Mean acid out- put, meq/24 hr | % increase |
|-----|--------|----------------------------------|----------------------------------|---------------|
| 73 | Before | 39 | 23.6 \pm 9.1* | 299 |
| | After | 42 | 94.1 \pm 26.5 | |
| 74 | Before | 35 | 25.8 \pm 14.1 | 295 |
| | After | 24 | 101.9 \pm 58.6 | |
| 95 | Before | 42 | 2.6 \pm 1.4 | 630 |
| | After | 30 | 19.1 \pm 8.0 | |
| 96 | Before | 40 | 1.4 \pm 1.2 | 849 |
| | After | 35 | 13.3 \pm 2.8 | |

* Stand. dev.

t for 4 sets = 15.40. P < .001.

vein drains into the upper cut end of the inferior vena cava, completely by-passing the liver, and the lower inferior vena cava drains into the upper cut end of the portal vein, maintaining a large flow of systemic venous blood to the liver. As soon as the dog was eating the same amount as before operation collections were again made and continued for 24 to 42 days.

The *results* are set out in Table I. Each dog showed a profound increase in mean acid output after portacaval transposition. There was generally an increase in both the volume and acidity of the juice secreted by the pouch. All the dogs lost weight slowly during the experiment.

Dog # 74, who was the smallest of the 4, became dehydrated and anorexic 5 days after transposition due to loss of large amounts of fluid and chloride in the pouch secretion. 1000 ml of 0.9% NaCl were given subcutaneously on each of 3 successive days and his appetite returned. On the 23d day after transposition dehydration and anorexia again occurred, parenteral fluids were not given, and he died the next day. Autopsy revealed no cause of death other than extreme dehydration. The mean acid output for Dog # 74 was calculated using all post-transposition collections including those low values occurring when the dog was eating poorly.

Dogs # 73 and # 95 died of perforating ulcers in their pouches 112 and 55 days after transposition, respectively. Dog # 96 is alive and well 42 days after transposition. The dogs that died had grossly normal livers, open portacaval anastomoses, and no ulcerations

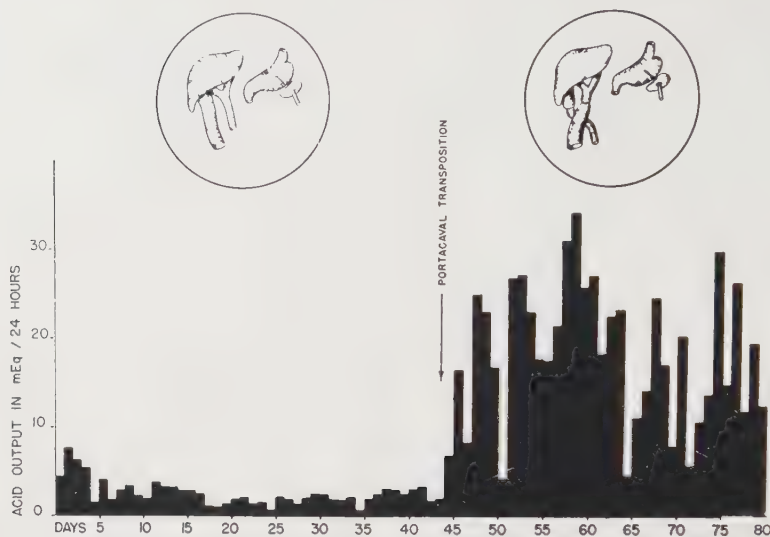


FIG. 1. Effect of portacaval transposition on secretion of Heidenhain pouch. Dog #95.

of the stomach or duodenum.

Discussion. Portacaval transposition was selected in this study because a method was desired which would provide a complete shunting of portal blood around the liver and still cause minimal impairment of hepatic function. Child and co-workers(8) found that hepatic regeneration was absent in dogs with Eck fistulae, and was good, though not entirely normal, in dogs with portacaval transpositions. Silen, *et al.*(9) concluded after careful studies that both laboratory and histologic findings in dogs with transpositions were very similar to those of normal animals. Tolerance to oral ammonium lactate was diminished both in dogs with Eck fistulae and in others with portacaval transpositions, but this was much more marked in the former. Thus, the observed increase in pouch secretion after transposition probably is not due to impairment of parenchymal liver function.

In forming a Heidenhain pouch the vagus nerves to the pouch are cut. The secretion from such a pouch is largely in response to humoral agents as evidenced by its low levels in fasting and after antrum resection(10). The increase in secretion found in these experiments therefore is of humoral origin. Among humoral substances which immediately come to mind are gastrin and ammonia. Gastrin is a potent hormone stimulating Hei-

denhain pouch secretion and it is conceivable that gastrin is inactivated in the liver. Ammonia is a substance which enters the portal blood from the digestive tract and is normally cleared from this blood by the liver. Since the dog food (Thoro Fed) eaten by these animals contains at least 10% crude protein, there was an adequate source for ammonia formation in the gut. However, the exact agent at work here to increase secretion after transposition and its anatomical site of origin remain to be determined.

Summary. 1) Heidenhain pouch secretion in dogs is markedly increased following portacaval transposition. 2) This may be due to increased effect, after shunting of portal blood around the liver, of a humoral secretagogue which originates in the abdominal viscera and is normally inactivated or excreted by the liver. 3) This finding offers a new approach to the cause of the ulcer diathesis exhibited by patients with cirrhosis of the liver.

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Physiological Aspects of Aging. I. Efficiency of Absorption and Phosphorylation of Radiothiamine.* (23664)

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Mills and associates(1,2) have reported that the thiamine requirement, in terms of dietary concentration, is approximately twice as great for old rats as for growing rats. Thiamine was found to be unique among the B vitamins in this respect. Friedemann *et al.* (3) observed that oral doses of more than 5 mg were poorly absorbed by humans, whereas many times this quantity of other water-soluble vitamins is efficiently absorbed, and Kirk and Chieffi(4) estimated that 50% to 75% of a dose of 5 mg of thiamine hydrochloride apparently was unabsorbed by either young or old subjects. In the present study the absorption of radioactive thiamine by rats 1 month to 2 years old, and by young rats in deficient, normal and supernormal states of thiamine nutriture was studied. As an index of the influence of aging on the conversion of thiamine to its phosphoric acid esters, and on the efficiency of the general phosphorylation mechanism, the radioactive compounds in the liver were partitioned into phosphorylated and unesterified forms which were estimated separately.

Methods. Albino rats of the Sprague-Dawley strain were maintained on a stock diet with a supplement of 2500 I.U. Vit. A per month. Individuals of various ages were

fasted for 24 hours and placed in a urino-fecal separator(5). An oral dose of 120 μ g of radiothiamine (thiazole-2-C¹⁴) with a specific activity of 6 μ c/mg was administered by stomach tube under light ether anesthesia. Urine and feces were collected for a 48-hour period; the animals then were sacrificed and the livers were removed and stored at -18°C. The feces were dried for 12 hours at 105°C, ground finely, and a 1 g sample was digested for 16 hours under toluene at 37°C, using 20 mg papain and 20 mg clarase in 25 ml 0.5% acetate buffer at pH 4.5. The hydrolysates were filtered through No. 1 Whatman paper and washed several times with distilled water. Duplicate 200 λ aliquots were plated for counting in a Tracerlab Q-gas windowless gas flow counter connected to a Nuclear scaler. The efficiency of the extraction procedure was determined by plating a fine suspension of the undigested residue and of the original ground samples, prepared by agitating vigorously for 5 minutes in a Waring blender. For a series of samples a recovery of at least 95% of the total fecal radioactivity was obtained in the filtrate. Hydrolysates of feces from 2 treated animals were chromatographed on filter paper as described below and radioautographs were prepared. Only one spot, at R_f .66, corresponding to that of thiamine, was detected, indicating that this was the sole radioactive compound present in significant amounts after hydrolysis. Separation of thiamine and its phosphoric acid esters in liver was achieved

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by a modification of the procedure of Westenbrink and Steyn-Parvé(6) for the release of diphosphothiamine (DPT) from protein and of Siliprandi and Siliprandi(7) for its chromatographic separation from thiamine and other phosphate esters. It has been shown (6) that DPT may be released from animal tissues without hydrolysis of the phosphate ester by placing the minced pulp in a water bath for 5 minutes in .01N HCl, whereas in 1N HCl DPT is converted to monophosphothiamine (MPT). In the present study 200 λ aliquots of the supernatant obtained from this procedure were placed on strips of pretreated Whatman No. 1 filter paper(8) which were developed in the ascending direction(7). DPT and thiamine, which were detectable under ultraviolet light after spraying the dried strips with thiochrome reagent, exhibited R_f values of .21 and .66, respectively. Heating DPT in 1N HCl gave rise to a thiochrome-reactive compound at R_f .37, corresponding to that of MPT(7). The concentrations of triphosphothiamine (TPT) and MPT, reportedly present in liver(9,10), were too small for detection on sample strips by the thiochrome reaction or by scanning. The sample strips were cut into 2 segments so as to separate thiamine from its esters, as indicated by the development of standard strips with thiochrome reagent. The segments were eluted with distilled water and 200 λ aliquots were plated. Evidence that the intestinal wall performs a regulatory function in thiamine absorption(3) and the suggestion that an impairment of thiamine absorption may occur in rats deficient in this vitamin(11), prompted a study of the absorption of radiothiamine in 7-week-old rats which had received inadequate, "normal" and supernormal amounts of thiamine in the diet. Three groups of 6 weanling rats were fed a synthetic thiamine-deficient basal diet. One group received no supplementary thiamine, a second received 2 μ g per g and a third 50 μ g per g of diet. The absorption tests were made, as described previously, when the deficient animals had begun to lose weight. In order to prevent wide disparities in body weight between groups, the weights of the rats fed the sup-

TABLE I. Excretion of C^{14} following a 120 μ g Oral Dose of Radiothiamine by Young Rats Maintained on Three Different Levels of Thiamine. 6 rats/series.

| Group | Body wt (g) | Thiamine pretreatment (μ g/g diet) | 48-hours C^{14} excretion (% of dose) in | |
|-------|----------------|---|--|----------------|
| | | | Feces* | Urine* |
| 1 | 85.5 \pm 1.4 | None | 4.3 \pm .5 | 14.7 \pm .6 |
| 2 | 85.5 \pm 2.3 | 2 | 5.2 \pm 3.3 | 11.9 \pm 2.8 |
| 3 | 91.5 \pm 3.8 | 50 | 8.8 \pm 2.8 | 28.3 \pm 1.2 |

* Mean and stand. error.

plemented diets were controlled by regulating their food supply so that all animals were of comparable size when the test was administered. Following the dose, all rats were given 4 g of the basal diet per day for the 2-day collection period.

Results. Absorption of the 120 μ g dose of labelled thiamine was found to be about 95% efficient in young rats and to be unaffected by the previous level of thiamine intake (Table I). However, individual animals exhibited considerably poorer absorption, the highest fecal counts being 18.9% and 22.8% of the dose, while in other cases absorption was essentially quantitative. The increased urinary excretion in group 3 probably is a reflection of greater tissue saturation resulting from the high thiamine intake.

Since it has been found(12,13) that a small quantity of radioactivity is excreted in the feces of the rat after injection of thiazole-2- C^{14} thiamine or S^{35} -thiamine, the data for fecal C^{14} excretion may slightly over-estimate the unabsorbed portion by the amount of the endogenous contribution. As a check, 6 young rats, previously fed a stock ration, were given 120 μ g of radiothiamine intraperitoneally and the feces were collected for 48 hours. Radioassay indicated that 0.3-1.4% of the injected activity was excreted in the feces, amounts in close agreement with those reported previously(12,13). The slightly higher value for fecal activity obtained for group 3 may be partially attributable to an increased endogenous contribution to the feces coincident with the increased urinary excretion.

The fecal excretion of C^{14} by rats of different age groups following the 120 μ g oral dose of radiothiamine is summarized in Table

TABLE II. Fecal Excretion of C^{14} by Rats of Different Ages 48 Hours after a 120 μ g Oral Dose of Radiothiamine.

| Age (mo) | No. rats | Body wt (g)* | 48-hr C^{14} excretion in feces (% of dose)* |
|----------|----------|--------------|--|
| 1- 2 | 23 | 94 \pm 6 | 4.8 \pm 2.8 |
| 5- 8 | 12 | 391 \pm 14 | 6.8 \pm 1.8 |
| 14-16 | 5 | 435 \pm 14 | 6.0 \pm 5.1 |
| 19-20 | 6 | 436 \pm 11 | 12.9 \pm 3.2 |
| 22-24 | 11 | 400 \pm 28 | 23.4 \pm 3.6 |

* Mean and stand. error.

II. No agewise effect is evident until the animals reached 19-20 months, when a reduction in absorption is indicated, and a further decrease is apparent in the 22-24-month-old group. In the rats up to 16 months old, absorption in several cases was essentially quantitative and in only 8 out of 40 observations was it less than 90%. In the two oldest age groups (19-24 months) 13 out of 17 animals absorbed less than 90% of the dose, the minimum values being 55.7% and 57.2%.

The results of the assays for C^{14} -thiamine and its esters in the liver are given in Table III. Significantly more radiothiamine ($P < .01$), expressed either as total disintegrations per minute or as % of absorbed radiothiamine, was found in the livers of the early mature rats than in those of the growing animals. However, this difference becomes insignificant when adjustment is made by covariance analysis for the disparity in body weight between the 2 groups. Among the adult animals, significantly less activity ($P < .05$) was found in both the esterified and unesterified fractions of the older group (22-24 months) when compared with the younger (5-8 months), after adjustment for differences in body weight and radiothiamine absorbed. A shift in the ratio of the vitamin

to its esters appears to accompany maturity, since 57% of the total activity was found in the esterified form in the livers of the growing animals and only 22% and 18% in the early mature and old rats, respectively.

Discussion. The above results indicate that thiamine is efficiently absorbed by rats up to an age of approximately 19-20 months, beyond which a substantial decrease in efficiency occurs. The single dose of labelled thiamine used may be regarded as about tenfold the minimum daily requirement for growth. Watanabe(14) has reported that young rats absorb doses of 120, 240 or 400 μ g of unlabelled thiamine with about the same efficiency, indicating that there is no threshold value within this range.

The postulation that the ability of thiamine-deficient rats to absorb this vitamin is impaired because of lesions of the gut wall (11) is not supported by the results shown in Table II. Magyar and Gábor(15) have reported that the absorption of thiamine from an isolated loop of intestine is reduced by prior administration of large doses of thiamine or other B vitamins, and they suggested that this effect is due to an exhaustion of the phosphorylation mechanism. No impairment of radiothiamine absorption was encountered in the present study after continuous administration of about 50 times the necessary dietary concentration of thiamine. The lower recovery of labelled thiamine in the livers of the aged rats may be attributable to a reduction in protein binding or to a faster turnover rate.

The influence of pathology on the outcome of this study, particularly as it may have affected the absorption of thiamine, is impossible to assess. Although some of the rats in the oldest age group were slowly declining in

TABLE III. Liver C^{14} Distribution in Rats of Different Ages 48 Hours after a 120 μ g Oral Dose of Radiothiamine.

| Age group (mo) | No. rats | Body wt (g) | DPM* ($\times 10^{-3}$) in liver as | | % of absorbed radiothiamine in liver as | |
|----------------|----------|--------------|---------------------------------------|-----------------|---|-----------------|
| | | | Thiamine | Thiamine esters | Thiamine | Thiamine esters |
| 1- 2 | 6 | 143 \pm 4† | 10.6 \pm 2.5 | 13.8 \pm 6.3 | .67 \pm .16 | .87 \pm .40 |
| 5- 8 | 10 | 408 \pm 19 | 43.2 \pm 7.6 | 12.2 \pm 2.8 | 2.97 \pm .51 | .83 \pm .18 |
| 22-24 | 8 | 342 \pm 12 | 20.0 \pm 1.4 | 4.4 \pm .8 | 1.50 \pm .12 | .33 \pm .06 |

* Disintegrations/min.

† Mean and stand. error.

weight there was no apparent correlation between recent weight changes and the efficiency of absorption. A number of animals were discarded because of tumors and others because of a chronic respiratory disease which was the most prevalent health problem.

Summary. Absorption of an oral dose of 120 μ g of C^{14} -labelled thiamine by the rat has been studied as a function of age and the state of thiamine nutrition. Approximately 95% was absorbed up to an age of 19-20 months, when the efficiency declined to about 75% at 22-24 months. Absorption by thiamine-deficient animals was not different from that of rats receiving normal or excess quantities of thiamine in the diet. The proportion of absorbed radiothiamine found in esterified and unesterified forms in the livers of rats 22-24 months old was less than that in rats 5-8 months old.

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Chlorpromazine Blockade of Water Imbibition by Frog Gastrocnemius. (23665)

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The imbibition of water by frog gastrocnemius muscle has been studied extensively by Halpern and Reuse(4) and by Courvoisier and Ducrot(1), in an attempt to determine the mechanism of action of various drugs, chiefly phenothiazine derivatives, in blocking water uptake by this tissue. These investigators were able to rule out antihistaminic and local anesthetic potency as actions capable of being correlated with water imbibition block. Van der Kloot(6) has measured rate of sodium extrusion from frog gastrocnemius in hyponatric media and has related this to cholinesterase activity, but has not correlated the phenomenon with water transport.

Since phenothiazine compounds possess other pharmacologic properties than those

mentioned above, it has seemed of interest to us to examine selected structures of this type for correlation of these activities with water imbibition block. In the course of this work, a number of additional non-phenothiazine compounds were used for activity analogy in elucidation of the phenomenon.

Methods. The technic was essentially that of Halpern and Reuse(4), modified as follows: Gastrocnemius muscles were removed as rapidly as possible after pithing of the frogs, rinsed in distilled water, blotted and weighed. They were then assigned at random to beakers containing 45 ml of distilled water containing either no drug or the drug under examination. The drugs were used in a final concentration of 0.0016 M as employed by

TABLE I. Influence of Drugs on Water Imbibition and Ion Loss.

| Compound | Salt | Molar- ity | Increment ratios | | | | | |
|-----------------------------|------------------------|------------------|------------------|-------|-------|-------|-------|-------|
| | | | 1 hr | | | 2 hr | | |
| | | | Wt | Na | K | Wt | Na | K |
| Chlorpromazine (17) | HCl | .0016 | 29.8 | 113.7 | 176.6 | 15.2 | 122.2 | 182.1 |
| " + ATP (3) | HCl - phosphate | " | 42.8 | | 305.8 | 30.7 | | 731.0 |
| Promazine (3) | HCl | " | 47.0 | 134.4 | 279.0 | 26.9 | 169.0 | 340.8 |
| CPZ + epinephrine* (3) | HCl + bitartrate | .0008 | 50.5 | 105.3 | 188.3 | 34.1 | 137.5 | 237.8 |
| Prochlorperazine (3) | Dimalate | .0016 | 51.1 | 120.4 | 221.1 | 55.0 | 125.8 | 208.1 |
| Chlorpromazine (6) | HCl | .0008 | 53.7 | 117.6 | 156.7 | 37.7 | 132.8 | 174.8 |
| Promethazine HCl (5) | Dihydrochloride | .0016 | 55.4 | 129.0 | 177.0 | 36.0 | 186.0 | 216.0 |
| Ethopropazine (3) | " | " | 62.6 | 122.6 | 197.9 | 37.9 | 116.0 | 218.7 |
| CPZ + Marsilid + (5) | HCl + phosphate + | .0008 | 64.4 | 129.3 | 153.8 | 53.2 | 157.7 | 187.2 |
| epinephrine* | bitartrate | | | | | | | |
| Methscopolamine bromide (5) | Bromide | .0016 | 65.9 | 113.0 | 97.0 | 70.8 | 129.0 | 111.0 |
| Phentolamine HCl (5) | HCl | " | 66.2 | 117.0 | 124.0 | 55.2 | 144.0 | 153.0 |
| CPZ + Marsilid (6) | HCl + phosphate | .0008 | 66.6 | 109.0 | 145.6 | 62.9 | 117.5 | 179.1 |
| Perphenazine (5) | HCl | .0016 | 69.8 | 123.0 | 171.0 | 85.1 | 140.0 | 233.0 |
| Chlorpheniramine (5) | Maleate | " | 73.8 | 88.0 | 109.0 | 66.5 | 149.0 | 135.0 |
| Dinitrophenol (3) | " | " | 80.1 | 97.7 | 131.0 | 73.4 | 97.3 | 152.3 |
| Phenoxybenzamine (2) | HCl | " | 81.1 | 101.0 | 123.0 | 115.1 | 107.0 | 152.0 |
| Azapetine (3) | Phosphate | " | 85.2 | 100.0 | 91.0 | 83.8 | 102.0 | 121.0 |
| Marsilid (6) | " | .0008 | 85.7 | 107.8 | 107.5 | 83.9 | 110.1 | 102.3 |
| Saline (5) | " | .0016 | 87.0 | | 103.0 | 97.8 | | 116.0 |
| Marsilid + epinephrine* (6) | Phosphate + bitartrate | " | 89.1 | 77.6 | 99.5 | 100.0 | 114.9 | 116.9 |
| Aminopyrine (5) | " | " | 88.2 | 108.0 | 105.0 | 93.0 | 147.0 | 126.0 |
| Marsilid (5) | Phosphate | " | 90.0 | 117.3 | 113.3 | 104.0 | 123.5 | 113.5 |
| Prednisolone (3) | Hemisuccinate | " | 92.5 | | 108.8 | 90.6 | | 107.2 |
| Phenoxybenzamine (3) | HCl | .0008 | 93.9 | 76.0 | 116.5 | 97.3 | 78.8 | 100.9 |
| Epinephrine (9) | Bitartrate | 10 ⁻⁷ | 96.2 | 109.3 | 107.4 | 100.0 | 109.7 | 116.0 |
| Acetazolamide (5) | " | .0016 | 102.1 | 102.3 | 120.4 | 91.5 | 84.9 | 101.7 |
| Phenylbutazone (5) | Na salt | " | 104.0 | | 98.0 | 107.2 | | 109.0 |
| Adenosine triphosphate (3) | Phosphate | " | 106.8 | | 128.9 | 99.4 | | 149.9 |
| Physostigmine (3) | Sulphate | " | 108.7 | 92.3 | 98.6 | 107.9 | 85.0 | 89.9 |
| Amphetamine (3) | " | " | 111.0 | 95.0 | 77.0 | 111.5 | 93.0 | 118.0 |
| Phenoxybenzamine (3) | HCl | .0032 | 114.3 | 128.8 | 181.2 | 133.3 | 111.5 | 161.7 |

No. in parentheses indicate No. of experiments.

* Molarity of epinephrine = 10⁻⁷.

CPZ = Chlorpromazine.

Courvoisier and Ducrot(1) except in a few cases wherein more concentrated or dilute solutions were examined. The 0.0016 Molar (0.05% approx.) concentration was found by these workers to be the most effective of those tried. Each beaker contained 2 muscles from different frogs. Samples of the medium (5 ml) were removed at zero time for sodium and potassium determinations by the use of a Beckman DU spectrophotometer equipped with flame photometry attachment. The filled beakers were then shaken at room temperature in air in a Dubnoff apparatus for 45 minutes after which time samples of medium were taken for ion determinations and the muscles were blotted and weighed in random sequence. They were then returned to the shaker and the weighing and sampling procedure repeated after an additional 45 minutes of shaking. The data were treated as follows: The weights

of the 2 muscles on any treatment were averaged and the percentage increment during the first and second hours calculated. This average percent increment was divided by that obtained from the control muscles in water alone and the result expressed as the following

$$\text{ratio: } \frac{\Delta \% \text{ with drug}}{\Delta \% \text{ without drug}} \times 100. \text{ It will be}$$

seen that absence of drug effect will result in a ratio close to 100. Since the initial ion concentrations were negligible, similar ratios for ion accumulation in the medium were calculated directly using the increments expressed in milliequivalents/liter rather than percentage increments. Thus the ratio becomes:

$$\frac{\Delta \text{ meq with drug}}{\Delta \text{ meq without drug}} \times 100.$$

Results. All of these results were ranked in order of increasing weight ratios in Table I.

Halpern has shown a lack of correlation of block of water imbibition with antihistaminic activity. This has been corroborated, since of the 3 antihistamines tested, chlorpromazine, promethazine, and chlorpheniramine, the latter although most potent in antihistaminic action, is least active in blocking water uptake in this preparation.

It will be seen that the least water imbibition occurred in muscles treated with chlorpromazine. Aside from the antihistaminic effect of this compound, among its most important actions are those of tranquilization, and adrenergic blockade. It would not appear that tranquilization is of import, since prochlorperazine and perphenazine are more effective as tranquilizers than is chlorpromazine [Irwin and Govier(5) Vischer(7)] but much less effective as blockers of water imbibition.

It would appear that if the blockade of water imbibition be concerned with adrenergic blockade, then epinephrine should be able to lessen the degree of block. However, addition of epinephrine (1×10^{-7} M) to beakers containing chlorpromazine (0.0008 M) did not reduce the block. Likewise, increase of epinephrine concentration to 0.0008 M was without result. In addition, as pointed out above, adrenergic blockers other than chlorpromazine are not impressive in their ability to inhibit water imbibition. It would appear that epinephrine should have been reasonably stable in this system, since we have been unable to demonstrate monamine oxidase activity in this preparation. Although the tissue was not examined for catacholase activity, the enzyme is apparently not often found in muscle, if at all.

Physostigmine at 0.0016 M did not alter water imbibition significantly.

Compounds characterized by carbonic anhydrase inhibition (acetazolamide), anticholinergic activity (methscopolamine bromide, ethopropazine), adrenergic blockade (promethazine, phentolamine, phenoxybenzamine, azapetine), analgetic-antiinflammatory activity (aminopyrine, phenylbutazone, prednisolone) adrenergic activity (amphetamine, epinephrine), monamine oxidase inhibition (Marsilid), cholinesterase inhibition (physo-

TABLE II. Correlation of Weight Gain with Ion Loss.

| | 1st hr | 2nd hr |
|-----------|--------|--------|
| Sodium | -.45 | -.58 |
| Potassium | -.66 | -.54 |

stigmine), metabolic uncoupling (dinitrophenol) and high energy phosphate activity (adenosine triphosphate) do not produce outstanding inhibition of water uptake.

In an effort to determine whether or not pH of the solutions was correlated with water uptake, pH was determined on most of the media used. These values varied between pH 3.2 and pH 6.85 and showed no correlation with drug effect. Additionally, when muscles were soaked in phosphate buffers covering part of this range with extension to the alkaline side, (pH 5.5 to pH 8.05) all at 1.6×10^{-3} molar, no significant differences were seen in water uptake as the pH was varied.

In order to determine whether or not a correlation exists between weight changes and increase of sodium or potassium in the medium, correlation coefficients were calculated for weight increase versus sodium and potassium loss. These are recorded in Table II.

All of these coefficients are significant at the 0.05 level. It would thus appear that water imbibition is related to ion transport in these experiments.

The observation that physostigmine did not block water imbibition, nor did it influence sodium or potassium loss, suggests that the frog gastrocnemius behaves differently in distilled water with regard to response to cholinesterase inhibition than in hyponatric Ringer as used by Van der Kloot. Determinations in our laboratories of the ability of chlorpromazine to inhibit specific (methacholine) and non-specific (butyryl choline) cholinesterase of frog gastrocnemius have shown the compound to be ineffective against the specific enzyme, but to produce 52.5% inhibition of non-specific cholinesterase at 1.6×10^{-3} M when substrate was used at 1×10^{-2} M.

Greig & Holland(2) and Greig & Gibbons (3) have reported that the presence and integrity of cholinesterase on cell membranes is necessary for the maintenance of membrane integrity and that chlorpromazine is able to

prevent removal of cholinesterase from red blood cells by lysolecithin. In an attempt to determine whether frog gastrocnemius loses cholinesterase on soaking in distilled water and whether chlorpromazine can block this loss, cholinesterase activity was determined manometrically on lyophilized, reconstituted water medium in which frog muscles had been immersed with and without chlorpromazine. The results demonstrated that if cholinesterase be leached out of the muscle, the amount is so small that it is impossible of determination by these technics. It was also found in characterizing the cholinesterase of frog muscle, that the absolute content of enzyme is exceedingly small.

Thus it would appear that no ready explanation is at hand for the ability of chlorpromazine and to a lesser extent certain other phenothiazines to inhibit water imbibition under these conditions, although correlation exists with increased cell membrane permeability to potassium and sodium.

No attempt has been made to determine the extent to which the drugs used are able to penetrate the frog muscle cell although differences in activity between such compounds as phentolamine, phenoxybenzamine, and azapetine suggest that permeability effects may have influenced these results to some extent.

Summary. 1. The chlorpromazine-induced

inhibition of water imbibition by frog gastrocnemius is unrelated to tranquilizing ability, adrenergic blockade, and antihistaminic activity. 2. Selected compounds exhibiting carbonic anhydrase inhibition, anticholinergic activity, analgetic and antiinflammatory activity, adrenergic activity, monamine oxidase inhibition, metabolic uncoupling, cholinesterase inhibition and high energy phosphate activity produce only minor or no inhibition of water uptake. 3. Correlation exists between block of water imbibition and loss of sodium and potassium from the muscle.

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Effects of Chemotherapeutic Agents on Metabolism of Human Acute Leukemia Cells *in vitro*. (23666)

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Cancer chemotherapy is currently directed chiefly towards chemical synthesis, animal screening, and clinical trial of antimetabolites. Some of these antimetabolites are designed to act primarily against specific biosynthetic reactions with the thought that they might function as anti-tumor agents by interfering with nucleic acid or coenzyme production in neo-

plasms. None of the chemotherapeutic drugs employed has universal applicability to patients with cancer. Even within a single cancer type such as acute lymphatic leukemia, response of a particular patient to a given antimetabolite is unpredictable. This unpredictability is particularly serious in those patients with fulminating neoplasms for whom prompt selection of the most effective agent offers the only hope of remission.

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With these problems in mind, a method has been devised for screening chemotherapeutic drugs that depends upon an evaluation of their effects on the glycolysis of leukocytes taken from leukemic patients. To our knowledge there have been no published reports on the effect of chemotherapeutic agents on overall metabolism of leukemic cells. Glycolysis has been chosen as an indicator of metabolic anti-tumor activity for several reasons. Of these, the most important is the key role of aerobic glycolysis as an energy source for tumors(1). This is a universal property of all cancer cells thus far studied, which we have recently confirmed for leukemic cells(2). Secondly, there is an observed parallelism between inhibition of tumor growth *in vivo* and glycolytic inhibition *in vitro*(3-5). In addition, methods have been developed in our laboratories for selectively inhibiting glycolysis at the level of the hexokinase reaction. Acute leukemia is ideally suited for such study for several reasons: This neoplasm is readily accessible and can be serially sampled, and selected material is almost devoid of "contaminating" tissue cells. Much clinical experience is available in regard to the natural history and response to chemotherapy of acute lymphatic and acute myelogenous leukemias. *In vitro* studies of the metabolism of isolated leukemia cells enables one to measure the cells' metabolic sensitivity to a battery of usual chemotherapeutic agents. The effects of these anti-leukemic drugs can be used as a standard against which to evaluate new, clinically untested but theoretically interesting materials.

Methods. Patients were selected who had high white blood cell counts, consisting chiefly of blast and abnormal cells. The blood sample was drawn in a heparinized syringe and transferred to a solution of heparinized fibrinogen in serum to increase the rate of sedimentation of the red blood cells(6). Using bovine fibrinogen (Armour), and banked human serum, the concentration of fibrinogen in the blood-serum mixture was raised to 8-9 mg/cc. This mixture was allowed to stand at room temperature for 15-45 minutes, and a favorable separation was achieved with 12-20 white blood cells per red blood cell in the superna-

tant. One cc aliquots of the supernatant were pipetted into Warburg manometer flasks, and glycolysis measured in the usual manner in the presence, and in some instances the absence, of air. The test vessels contained the white cell suspension to which had been added glucose at 2 mg/cc, sodium bicarbonate at 1.5 mg/cc and the chemotherapeutic drug under analysis. The anaerobic determinations measured only the process of acid formation from glucose. Summerson manometers were used to determine aerobic glycolysis, O_2 uptake, and CO_2 production by a single sample. In the presence of air, the observed manometric (h) readings during the experimental period represented the algebraic sum of gas pressure changes due to 3 processes: (1) CO_2 liberated from bicarbonate by the lactic and other acids formed in glycolysis, (2) CO_2 formed by respiration and (3) negative component due to O_2 uptake through respiration. These 3 components were quantitatively distinguished by means of the Summerson differential manometer. Thus far, 8 patients with acute lymphatic leukemia, 5 with acute myelogenous leukemia, and 4 non-leukemic patients with leukocytosis were studied, as well as several cases of chronic leukemias not to be detailed here. Several examples of the data obtained with acute leukemia cells are cited as representative of the general experience with such cases. Repeat experiments done when possible generally showed good reproducibility, provided that the clinical state remained relatively stable.

Results. Table I illustrates the effects of methotrexate (amethopterin, purified by Dr. M. C. Li) and 6-mercaptopurine on the metabolism of acute lymphatic leukemia cells, taken from a 4-year-old boy. The peripheral blood contained 163,000 white blood cells/cmm comprised of 96% blast cells. The bone marrow was almost totally replaced with leukemic cells.

Anaerobic glycolysis, expressed as cmm of acid (CO_2 equivalent) produced per mg dry weight[†] per hour ($Q_A^{N_2}$), was 28. Anaerobic glycolysis of cells from acute lymphatic and

[†]1 mg dry weight averaged 14 million cells in numerous determinations made in the course of these studies.

TABLE I. Effects of 6-Mercaptopurine and Methotrexate on Metabolism of Acute Lymphatic Leukemia Cells.*

| Metabolism (mm ³ /mg dry wt/hr) | Control | 6-MP (0.1 mg/cc) | MTX (0.1 mg/cc) |
|---|-------------|---------------------|--------------------|
| Anaerobic glycolysis ($Q_A^{N_2}$) | 28 † | 28 | 28 |
| Aerobic " ($Q_A^{O_2}$) | 9.8† | 5.4 | 3.7 |
| Oxygen consumption (Q_{O_2}) | -4.8 | -5.6 | -6.3 |
| CO ₂ production (Q_{CO_2}) | 1.3 | 2.7 | 6.3 |
| Respiratory quotient (Q_{CO_2}/Q_{O_2}) | - .27 | - .48 | -1.0 |
| Aerobic glycolysis | | | |
| Respiration ($-Q_A^{O_2}/Q_{O_2}$) | 2.1 | 1.0 | .6 |
| "Metabolic type" | "Malignant" | "Benign" | "Normal" |

* 4-year-old male. WBC 163,000/mm³, 97% blast cells.

† Inhibited approximately 80% by hydrocortisone, triethylene thiophosphoramidate and 6-azauracil (0.2-0.5 mg/cc).

Measurements in human serum with added heparin (8 mg %), bovine fibrinogen (6 mg/cc), 0.2% glucose, 0.15% NaHCO₃, 5% CO₂ in air or N₂, pH 7.4, 37°C, 3 hr.

acute myelogenous leukemias was found to range from 20-30. The anaerobic glycolysis of cells from this patient with acute lymphatic leukemia was unaffected by incubating with methotrexate and 6-mercaptopurine at a concentration of 0.1 mg/cc fluid. Aerobic glycolysis ($Q_A^{O_2}$), however, was lowered by 6-mercaptopurine from 9.8 to 5.4, and even more by methotrexate to 3.7. This has been the average pattern observed in our study to date with acute lymphatic leukemia cells, namely inhibition of aerobic glycolysis by 6-mercaptopurine and greater inhibition by methotrexate, the latter causing an inhibition by as much as 80%. The inhibitions obtained were relatively independent of concentrations varied from 0.1 to 0.5 mg/cc and more.

The oxygen consumption (Q_{O_2}) of these cells was 4.8, and in other cases the general range was 4-8 in acute leukemias. Oxygen consumption was somewhat stimulated by 6-mercaptopurine and methotrexate, from 4.8 to 5.6 and 6.3 respectively. The respiratory carbon dioxide production (Q_{CO_2}) was low, 1.3, and was raised to 2.7 and 6.3 by 6-mercaptopurine and methotrexate respectively. It is of interest that the respiratory quotient (Q_{CO_2}/Q_{O_2}) of these lymphoblasts was of extremely low magnitude, -0.27. In other experiments the respiratory quotients varied widely from -0.8 to positive values.

The ratio of aerobic glycolysis to respiration ($Q_A^{O_2}/Q_{O_2}$) for these acute lymphoblasts

was found to be 2.1, a value in the range typical of cancer tissues (>1-10) as described by Burk(7). In the presence of 6-mercaptopurine this ratio was lowered to that seen with benign tumors (~1), and with methotrexate it was decreased to values generally found with non-cancerous tissues (<1-0).

The first example of acute myelogenous leukemia cells presented here (Table II) is that of a 58-year-old woman with a white blood cell count of 264,000, 75% of which were blast and abnormal cells. Aerobic and anaerobic glycolytic and O₂ consumption rates were comparable to those of the lymphatic case. The chief difference was a 3-fold increase in respiratory CO₂ production which in turn was reflected by an R.Q. of -0.71. This R.Q. is in the normal range of mammalian cells and contrasts with the lower value found in the lymphatic case cited. No effects on glycolysis or respiration were noted with methotrexate at concentrations as great as 1 mg/cc, or 10 times the concentration used to obtain inhibitory effects in the previous case. Furthermore there was no inhibition of aerobic glycolysis with either 6-mercaptopurine or 6-mercaptopurine plus methotrexate. No change was observed in the R.Q. upon addition of methotrexate.

The second case of acute myelogenous leukemia presented in Table II is that of a 51-year-old male with a white blood cell count of 334,000/cmm. The figures for anaerobic glycolysis, aerobic glycolysis and respiration

TABLE II. Effects of Methotrexate on Metabolism of Acute Myelogenous Leukemia Cells.

| Metabolism (mm ³ /mg dry wt/hr) | Control* | MTX (1 mg/cc) | Control§ | MTX (1 mg/cc) |
|--|-------------|------------------|-------------|------------------|
| Anaerobic glycolysis (Q ^N ₂) | 21 † | 21 | 20 ‡ | 20 |
| Aerobic " (Q ^O ₂) | 12 † | 12 | 7.8 | 8.2 |
| Oxygen consumption (QO ₂) | -5.6 | -5.3 | -6.3 | -6.3 |
| CO ₂ production (QCO ₂) | 4.0 | 3.7 | 4.9 | 4.9 |
| Respiratory quotient (QCO ₂ /QO ₂) | - .71 | - .69 | - .78 | - .78 |
| Aerobic glycolysis Respiration (-Q ^O ₂ /QO ₂) | 2.2 | 2.3 | 1.2 | 1.3 |
| "Metabolic type" | "Malignant" | "Malignant" | "Malignant" | "Malignant" |

* 58-yr-old female. WBC 264,000/mm³, 75% blast and abnormal cells.

† Not inhibited by 6-MP or 6-MP and MTX.

‡ Not inhibited by 6-MP, hydrocortisone, triethylene thiophosphoramidate or 6-azauracil (0.2-0.5 mg/cc).

§ 51-yr-old male. WBC 334,000/mm³, 81% blast and abnormal cells.

are very similar to those observed in the previous patient. Furthermore, there was again no appreciable effect on these functions when the cells were incubated with methotrexate. These 2 studies are representative of the values seen with acute myelogenous leukemia.

The metabolism of leukocytes from 4 non-leukemic patients with leukocytosis was studied. These cells *in vitro* were found to have high rates of aerobic glycolysis, in agreement with previous reports(8-10) on human intact leukocytes and leukocyte homogenates, but different from rat leukocytes where aerobic glycolysis is quite small(11). Anti-leukemic agents such as methotrexate, 6-mercaptopurine, hydrocortisone and 6-azauracil had no significant effect on the *in vitro* aerobic glycolysis of cells taken from 3 of these patients. In respect to aerobic glycolysis and response to drugs, these cells were grossly indistinguishable from those of acute myelogenous leukemia, so far as studied.

Discussion. It is believed that experiments of the type described here represent the first systematic study of the metabolic effects of chemotherapeutic agents on relatively pure human leukemic cells *in vitro*. The question of whether or not leukemic cells are cancer cells in the metabolic sense seems to be resolved by the observation that these acute leukemic blast cells exhibit significant amounts of aerobic glycolysis if incubated under "physiological" *in vitro* conditions. This question will be covered more fully in a later publication.

It has been indicated that reproducible metabolic changes occurred when leukemic cells were incubated with certain chemical agents. The metabolic response appeared to be a function of the cell type. Thus it was observed that agents such as methotrexate, 6-mercaptopurine, hydrocortisone, triethylene thiophosphoramidate, and 6-azauracil (1,2,4 triazine-3,5-dione)§ usually inhibited aerobic glycolysis of acute lymphatic leukemia cells. The latter 3 drugs also inhibited glycolysis of cells under anaerobic conditions. On the other hand, blast and other abnormal cells from acute myelogenous leukemic cases were particularly resistant to glycolytic inhibition by these same anti-leukemic agents. None of the 5 patients with acute myelogenous leukemia studied showed any appreciable metabolic effect with methotrexate. These observations are consistent with clinical impressions on the value of methotrexate in inducing remissions in acute lymphatic leukemia and the rarity of its beneficial effects in acute myelogenous leukemia. 6-mercaptopurine, and 6-mercaptopurine plus methotrexate, have shown occasional inhibitory effects on aerobic glycolysis in the cases of acute myelogenous leukemia studied. This finding is consistent with the use of 6-mercaptopurine as the "drug of choice" in treatment of this disease. Extensive correlative studies must necessarily await careful follow-up evaluations with a large group of such patients.

§ 1,2,4 triazine-3,5-dione was kindly supplied by Dr. Arnold Welch.

Preliminary studies with leukocytes from 3 non-leukemic patients with leukocytosis indicated that these cells were similar to those of acute myelogenous leukemia with regard to lack of glycolytic inhibition by anti-leukemic agents.

The respiratory quotients of acute lymphatic cells were often unlike those of any other known human cells, in that carbon dioxide production in relation to oxygen consumption was extraordinarily low, indeed at times CO_2 was apparently consumed (positive R.Q.). It can be seen that the effect of 6-mercaptopurine was to alter the R.Q. in the direction of normalization, and that methotrexate effected a similar and greater change in R.Q., to values as high as -1.0 .

Despite the demonstrated lack of glycolytic inhibition of acute myelogenous leukemia cells by drugs currently used in therapy of this disease, it is desirable to point out that we have found compounds that do profoundly depress glycolysis of such drug resistant cells (2,5,14-16). Certain glucose analogues, of which 2-desoxyglucose and 2-desoxygalactose are examples, are very potent glycolytic inhibitors. These are not only effective against leukemic cells (12) but also against K-2 carcinoma ascites cells and other tumor cells (12,13). It has been observed that folic acid, teropterin, methotrexate, and 6-mercaptopurine may also be very active glycolytic inhibitors of leukemia cells and ascites cells. Hochstein in our institute has demonstrated (14) that a profound glycolytic inhibition can be obtained in mouse melanoma mitochondria with methotrexate or 6-mercaptopurine, and that this can be overcome by increasing the concentration of ATP in the media, or by substituting glucose-6-phosphate for glucose as substrate. The localization of primary site of action on the mitochondrial hexokinase reaction in this non-ATP synthesizing system, together with evidence found on the similarity of action of folic acid and methotrexate in inhibiting glycolysis in certain cancer cell types, throws a new light on the question of the mode of action of these "anti-metabolites." We have proposed that all of these structur-

ally related compounds act by interfering competitively with ATP in glucose phosphorylation by the hexokinase reaction.

Summary. 1. A method for studying the effects of known anti-leukemic agents on the glycolysis of intact leukemic leukocytes is reported. The possible applications of this method in evaluating other new and theoretically promising agents, and in selecting the most active agent in a particular clinical case, are indicated. 2. Methotrexate and 6-mercaptopurine inhibited the aerobic glycolysis of acute lymphatic leukemia cells. Methotrexate did not inhibit the equally large aerobic glycolysis of acute myelogenous leukemia cells, and 6-mercaptopurine did so only rarely. These metabolic findings parallel general clinical experience. 3. The bearing of these findings on the mechanism of action of certain cancer anti-metabolites (anti-folics and anti-purines) is pointed out.

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In vitro Sensitivity of Some Bacteria, their L Forms and Pleuropneumonia-Like Organisms to Antibiotics.*† (23667)

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Data on the sensitivity of pleuropneumonia-like organisms (PPLO) to various antibiotics are available in the literature(1,2). No such data have been available for the L forms of bacteria. Comparison of the two groups in this respect is of interest because they present many similarities, one of which is their marked resistance to penicillin. Such resistance is present in the L forms regardless of the sensitivity of the parent organism.

Materials and methods. The following bacteria and their L forms were studied: 3 Group A streptococci (AED, GL8, ADA),§ a Group C streptococcus (C), and *alpha*-hemolytic streptococcus (#23), a diphtheroid strain (NMI), 2 *Proteus* strains (52 and 18),|| a *Salmonella typhimurium* (TM), 2 *Vibrio* strains (EZ5 and Nankin)|| and an unidentified Gram negative bacillus (City).¶ The L forms were isolated on agar plates by ex-

posure to penicillin and maintained by serial transfer on agar plates containing penicillin (1000 units per ml) until transplanted for antibiotic sensitivity. Eleven strains of pleuropneumonia-like organisms were studied. Strains Campo, 4330, 1454, 9358 and Washington** were isolated from human genitourinary tracts. Strain K5 was isolated from an epizootic of goats.†† Preston‡‡ was isolated from a spontaneous polyarthritis of rats and A28 from a rat in our laboratory. 'L', ALG and C15 are saprophytic strains isolated from compost and well water.§§ Sensitivities were determined on agar plates. Solutions of the commercial antibiotics bacitracin, erythromycin, neomycin sulfate, chloramphenicol, chlortetracycline HCl, tetracycline HCl, oxytetracycline HCl, streptomycin sulfate and penicillin potassium were preserved in the frozen state until used. Appropriate amounts of the antibiotics were added to the medium before pouring the plates. Final concentrations of the antibiotics were 2, 5, 10, 20, 50, 100, 200, 400, 600, 1000 and 2000 micrograms per ml (or units for bacitracin and penicillin). The test plates were inoculated with L forms and PPLO by agar blocks cut out from 48- to 72-hour cultures. This procedure gave more uniform growth for these organisms than transfer from broth cultures. The bacteria were transferred to the test plates from 18-hour broth cultures. Examination for bacterial growth was made at 24 and 48 hours. Growth of the PPLO and of the L cultures was examined at 48, 72 and 96 hours with the hand lens and checked by

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§ Strains were those reported by Sharp, *et al.*(3).

|| *Proteus* strain and the *Vibrio* strains were kindly supplied by Dr. Robert Tulasne, Strasbourg, France. *Vibrio* EZ5 was isolated from water. Nankin is an old strain of *Vibrio cholerae* from the collection of the Pasteur Institute.

¶ Strain 'City' has not been finally classified. It is a Gram negative bacillus which occurred as a contaminant on an agar plate and which produced L forms abundantly. These L forms are similar to those of *Proteus*, but the bacillus does not swarm and it gives a negative urease reaction.

** Received from Dr. Ruth G. Whittler, Washington, D.C.

†† Received from Dr. H. E. Adler, Davis, Calif.

‡‡ Received from Dr. Wm. S. Preston, Ann Arbor, Mich.

§§ Received from Dr. Otto Kandler, Munich, Germany.

means of stained agar preparations. Sensitivity of the organisms was expressed as the lowest concentration of the antibiotic inhibiting growth. At least 2 tests were made with every organism and antibiotic at different times using different batches of media and only slight differences were observed between the tests. The procedure employed determines the inhibition of growth. However, as the plates used for any experiment were prepared and inoculated simultaneously, the differences observed between the various organisms are thought to represent real differences in their behavior to the antibiotics.

Results. The results are presented in Tables I and II. A conspicuous resistance comparable to that observed with penicillin is

not present with either the PPLO or the various L forms toward any other antibiotic tested. The sensitivity of the L forms isolated from various bacteria to different antibiotics is quite variable and usually parallels the sensitivity of the parent bacterium. An exception is bacitracin to which the L forms of streptococci and of *Vibrio* strain EZ5 are noticeably less sensitive than the parent bacterium. The enteric bacteria and their L forms are more resistant to the antibiotics than the other species studied and sometimes the bacteria, sometimes the L forms are slightly more resistant.

The sensitivities of PPLO strains isolated from man and animals are remarkably uniform. Their resistance to bacitracin and

TABLE I. *In Vitro* Sensitivity of the Bacteria and Their L Forms to Various Antibiotics. Concentrations are expressed as $\mu\text{g/ml}$ (or units for bacitracin and penicillin) and represent lowest inhibitory concentration.

| Organism | Bac. | Erythro. | Neo. | Chloro. | Tetra. | Oxytet. | Chlortet. | Strep. | Pen. |
|---------------------|-------|----------|------|---------|--------|---------|-----------|--------|---------|
| <i>Streptococci</i> | | | | | | | | | |
| ADA Bact. | <2 | <2 | 10 | <2 | <2 | <2 | <2 | 10 | <2 |
| L | 50 | " | " | " | " | " | " | " | 20,000 |
| GI-8 Bact. | <2 | " | " | " | " | " | " | " | <2 |
| L | 50 | " | <2 | " | " | " | " | <2 | 10,000 |
| AED Bact. | <2 | " | 10 | " | " | " | " | 10 | <2 |
| L | 400 | " | " | " | " | <5 | " | " | 10,000 |
| C Bact. | <2 | " | 100 | 5 | 5 | 5 | 5 | " | <2 |
| L | 50 | " | <2 | <2 | <2 | <2 | <2 | 5 | >20,000 |
| #23 Bact. | <2 | " | 10 | " | " | " | " | 10 | <2 |
| L | 50 | " | " | " | " | " | " | " | 20,000 |
| <i>Diphtheroid</i> | | | | | | | | | |
| NMI Bact. | 10 | 5 | 100 | " | " | " | " | 50 | * 10 |
| L | 20 | <2 | " | 5 | " | " | " | 100 | 20,000 |
| <i>Proteus</i> | | | | | | | | | |
| 18 Bact. | 400 | 400 | 200 | 100 | 100 | 200 | 200 | 200 | * 20 |
| L | 200 | 5 | 5 | 50 | 20 | 50 | 50 | 20 | 10,000 |
| 52 Bact. | 100 | 100 | 50 | 50 | 200 | 400 | 200 | 50 | * 50 |
| L | 1,000 | 600 | 600 | 200 | " | " | 600 | 200 | 20,000 |
| <i>Salmonella</i> | | | | | | | | | |
| TM Bact. | " | " | 2 | 10 | 20 | 20 | 20 | 20 | * 50 |
| L | " | " | 600 | " | 200 | 200 | 200 | 200 | 20,000 |
| <i>Unidentified</i> | | | | | | | | | |
| City Bact. | 600 | " | 50 | <2 | 50 | 100 | 50 | 50 | * 50 |
| L | 20 | 20 | 10 | " | 10 | 50 | " | 10 | 20,000 |
| <i>Vibrio</i> | | | | | | | | | |
| EZ5 Bact. | <2 | <2 | <2 | " | <2 | <2 | <2 | <2 | |
| L | 600 | " | " | 5 | " | " | 10 | " | 10,000 |
| Nan. Bact. | 400 | " | " | <2 | " | 10 | 20 | 20 | |
| L | 600 | " | " | 5 | " | <2 | 5 | <2 | 10,000 |

Bac. = bacitracin; Erythro. = erythromycin; Neo. = neomycin; Chloro = chloramphenicol; Tetra. = tetracycline; Oxytet. = oxytetracycline; Chlortet. = chlortetracycline; Strep. = streptomycin; and Pen. = penicillin.

* These determinations were made at a different time from those relating to the L forms.

TABLE II. *In Vitro* Sensitivity of Some Pleuropneumonia-like Organisms to Various Antibiotics. Antibiotics and their concentrations same as in Table I.

| Organism | Bac. | Erythro. | Neo. | Chloro. | Tetra. | Oxytet. | Chlortet. | Strep. | Pen. |
|------------|------|----------|------|---------|--------|---------|-----------|--------|---------|
| Campo | 600 | 200 | 10 | 10 | <2 | <2 | <2 | 20 | >20,000 |
| 4330 | " | " | " | " | " | " | " | " | 20,000 |
| 1454 | " | " | " | " | " | " | " | " | " |
| 9358 | 400 | " | " | " | " | " | " | 10 | " |
| Washington | 600 | 100 | <2 | <2 | " | " | " | " | " |
| K5 | " | 200 | 10 | " | " | " | " | 20 | >20,000 |
| Preston | " | " | " | 10 | " | " | " | " | " |
| A28 | " | " | 20 | " | " | " | " | " | 20,000 |
| Alg.* | " | <2 | <2 | <2 | " | " | 10 | <2 | " |
| C15* | " | " | " | 5 | " | " | " | " | " |
| 'L'* | " | " | " | " | " | " | " | " | " |

* Saprophytic strains.

erythromycin is fairly high, comparable to that of Gram negative bacteria. The saprophytic PPLO strains are sensitive to erythromycin and are more sensitive than the parasitic strains to neomycin, chloramphenicol and streptomycin also. On the whole, the sensitivity of PPLO to the various antibiotics is between the limits observed with the bacteria and their L forms. For example, the sensitivities of the L forms of the 2 *Vibrio* strains and of the saprophytic PPLO strains were similar toward all antibiotics tested. The PPLO, like the L forms, present an exceptional behavior toward penicillin only.

The similar reaction of the 2 groups to penicillin, like their morphological similarities, is probably the result of a common structural property, the lack of a rigid membrane comparable to the bacterial cell wall(3,4). Considering this and various biochemical observations, it has been inferred that the effect of penicillin may be to interfere with the production of the cell wall(3,5,6). On the other hand, the general similarity of behavior to various antibiotics of the PPLO, the L forms of bacteria and the bacteria themselves contributes to the concept that the PPLO are closely related to bacteria.

It is of interest that while penicillin makes possible the isolation of L forms, we had no such success with other antibiotics even when the difference between the sensitivity of the bacteria and their L forms is marked. For example, we were not able to obtain L forms with bacitracin from bacteria whose L forms grew well on media containing amounts of the antibiotic inhibitory for bacteria. Bacitracin

also inhibited the development of L forms when it was added in subinhibitory doses to plates containing penicillin. Apparently, the bacitracin in concentrations which allow the growth of L forms interferes with the transformation of bacteria into such forms. The initial stage of the development of L colonies, the growth of small granules from the large bodies, was observed in typhoid bacilli in the presence of streptomycin and chloramphenicol, but development stopped at this stage (7). In contrast to these antibiotics, a few amino acids, especially glycine, are good producers of L forms, although there is no wide difference between the concentrations inhibiting growth of bacteria and L forms.

Summary. The sensitivity of PPLO strains of various origin and of several bacteria and their L forms were examined in the presence of 9 antibiotics. The sensitivity of L forms to the various antibiotics with the exception of penicillin was comparable to that of the parent bacterium. However, the L forms of streptococci and of one *Vibrio* strain were consistently less sensitive to bacitracin than their bacterial forms.

All PPLO strains, like the L forms, were highly resistant to penicillin. The sensitivities of the parasitic strains to the various antibiotics were uniform; they were markedly resistant to bacitracin and erythromycin, like some bacteria. The 3 saprophytic strains were noticeably less resistant than the parasitic strains to several antibiotics, especially to erythromycin. The sensitivities of all strains of PPLO, of bacteria and of L forms of these bacteria were on the whole comparable.

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Demonstration of Myosin in Human Striated Muscle by Fluorescent Antibody. (23668)

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The antigenic property of myosin provides an opportunity to study this protein using immunological technics. Kesztyüs, Nikodemusz and Szilagyi demonstrated that myosin prepared from rabbit skeletal muscle was antigenic in dogs(1). Ebert has shown that chicken cardiac myosin produced antibodies in rabbits and that by the precipitin reaction it was possible to correlate the time of appearance of cross striations of the heart muscle with the presence of myosin(2). More recently Finck, Holtzer and Marshall(3) applied Coons' fluorescent antibody technic for localization of myosin in the chick muscle. This technic depends on selective binding of specific, fluorescent antibody to antigen present in tissue sections. The site of localization of antigenic substances within the morphological structures can be then demonstrated using fluorescence microscopy.

Our investigation represents a study of distribution of myosin in striated muscle by fluorescent antibody technic. It is the purpose of these studies on the localization of myosin in normal muscle to establish a basis of comparison for further study on the behaviour of myosin in diseased fibers and thus bring some insight into the problem of neuromuscular disorders.

Materials and methods. Preparation of antigen: Myosin was prepared from muscle of rabbit and goat and from human muscle, obtained on autopsy performed less than 2

hours post mortem. The muscle (*M. psoas* in most cases) was kept in the deep freeze until needed, *i.e.* for a period from several days to several weeks. The preparation followed closely the procedure of Szent-Gyorgyi(4). According to this method, the muscle is extracted with 0.6 M potassium iodide (KI) containing sodium thiosulphate after preliminary washing with water and with .05 M KCl to remove the muscle's adenosine triphosphate (ATP). The myosin was precipitated by diluting the extract with water to a KI concentration of .025 M. The precipitate was washed with .025 M NaCl and dissolved by adding NaCl to .5 M. The myosin was purified by 3 precipitations, and was dissolved in .5 M NaCl. Heavy impurities were removed by ultracentrifugation for 1 hour at 39,460 rpm. The myosin solution was passed through a Seitz filter before using it in precipitin reactions. The purity of the myosin preparations was ascertained in the ultracentrifuge. All operations were carried out in the cold. In addition, actin content was estimated by the method of Szent-Gyorgyi(4) from the change of the logarithm of the relative viscosity on addition of ATP. Most preparations contained less than 5% proteins other than myosin. Some myosin preparations were stored at -20°C in presence of .5 M NaCl and 50% glycerol. For immunizing rabbits and for precipitin reaction the glycerol was removed and the myosin precipitated by dilution or

TABLE I. Method of Assay of Anti-Human Myosin Rabbit Sera.

| Tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|--|------|-------|------|------|------|------|------|------|------|
| (A) Anti-myosin rabbit serum | | | | | | | | | |
| Dilution of serum | 0 | 0 | 0 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 |
| ml of antiserum transferred | | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 |
| ml NaCl—0.5M | .5 | .5 | | .5 | .5 | .5 | .5 | .5 | .5 |
| Myosin (1 ml 10 mg) | .5 | | .5 | .5 | .5 | .5 | .5 | .5 | .5 |
| Precipitation after 24 hr— 5°C (valued 0-4.0) | | | 3. | 2. | 1.5 | 1. | | | |
| mg of N/ml (Kjeldahl) of myosin, serum & washed precipitates | .79* | 5.69† | .99 | .69 | .29 | .22 | .16 | .18 | .16 |
| (B) Control normal serum—no precipitation | | | | | | | | | |
| mg of N/ml | .84 | 4.38 | 5.66 | 3.26 | 2.21 | 1.51 | 1.19 | 1.03 | 1.03 |
| Cal. nitrogen | | | 5.22 | 3.03 | 1.93 | 1.38 | 1.11 | .97 | .90 |

* Myosin.

† Serum.

dialysis. Such preparations proved also to be as satisfactory for immunizing rabbits and for precipitin reactions as fresh ones. *Assay of rabbit antibody to human myosin:* Antibody to human myosin was developed in 3 rabbits by intravenous injections of 8-10 mg of myosin in 0.5 M NaCl. Injections were given on alternate days for a period of 2 weeks. Five series of 6 injections each were administered allowing a period of rest between each series. The animals were bled from time to time, the serum removed, assayed for antimyosin antibody and the results compared with that of normal serum. The sera from 3 rabbits injected with myosin were assayed in 2-fold serial dilution from zero to 256 fold. Aliquots of 0.5 ml were transferred successively to tubes to which were added 2.5 mg of the antigen in 0.5 M NaCl solution. The degree of precipitation of the antigen-antibody reaction was evaluated from 0 to 4 and recorded immediately after preparation. The tubes were inverted several times and placed in the ice box at 5°C overnight. After 20 minutes at room temperature the amount of precipitate was again evaluated and recorded as the final reading. The procedure is shown in Table I for one lot of serum. In this assay dilution of antiserum was limited to 1:64 because differences in nitrogen below this dilution could not be determined. The reliability of this type of estimation of precipitation values was verified in some experiments by nitrogen determinations of washed precipitates and these values are compared with nitrogen of the control tubes containing no precipitates. Table

I shows mg of nitrogen/ml (Kjeldahl) of twice washed precipitates and the estimated values obtained by visual observations of the density of the precipitates. For comparison a similar series of tubes using normal serum were prepared concurrently. These gave negative results. In addition, the nitrogen values of the aliquots of the serum sample and of the antigen aliquot were also obtained. In the assay of normal control serum no precipitation occurred and the values of the nitrogen per ml would be expected to represent the value of antigen plus that of normal serum aliquot. The actual nitrogen values obtained from this series conform closely to the calculated values (Table I). These data lend support to the method of evaluating the titre of the sera by the observed density of the precipitates. A tube containing serum and saline but no antigen, and another tube containing myosin and diluent were added to each series as controls. The slight cloudiness of these tubes could readily be distinguished from the antigen-antibody precipitates. The highest concentration of myosin which would readily produce precipitation without inhibition of the antibody was adopted for the assay. Fig. 1 shows this to be between 1.25 and .625 mg for the sera of all 3 immunized rabbits. The rise in the titre of antibody to human myosin following intravenous administration (approximately 10 mg per dose) in the rabbit was determined by assay of the antisera in serial dilution for a period of over 6 months. Fig. 2 shows the relationship of this rise to the periods of successive inocula-

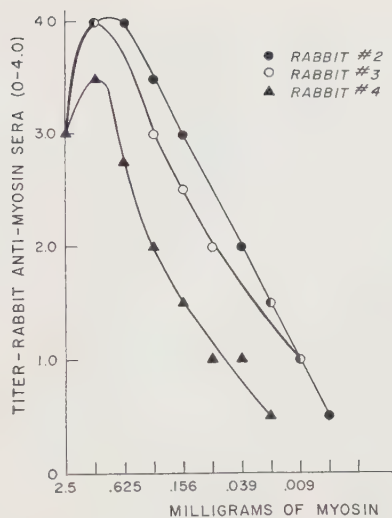


FIG. 1. Determination of equivalent zone between antigen and antibody showing the concentration of antigen (1.25 to 0.625 mg) required to give maximum precipitation with anti-human myosin sera from 3 rabbits.

tions, the rapid fall in titre with an extensive rest period of 2 months and an anamnestic rise in antibody titre with resumption of inoculations. A sample of anti-human myosin rabbit serum was mixed in serial 2-fold dilutions with serial dilutions of 3 myosin preparations derived from human, rabbit and goat muscle respectively. It was found that myosin of all 3 species reacted equally with antiserum of human myosin, as judged by the amount of precipitate in comparable tubes. The end points were also the same for all 3 myosin preparations tested. It will be noted that the species studied belong to 3 different orders of the mammals.

Preparation of fluorescent globulin conjugates: Following the determination of the titre of the rabbit antihuman myosin sera the globulin fractions were removed, pooled and conjugated to fluorescein isocyanate as described by Coons and Kaplan(5). The conjugated fluorescent globulin solution was dialyzed at 5°C against 0.15 M NaCl buffer, pH 7.4 to remove excess fluorescein. It was further purified by 2 ammonium sulfate fractionations at 5°C. After dialysis the fluorescent conjugate was further purified by ethanolic fractionation at -20°C. The precipitate was centrifuged down, the supernatant

removed and the precipitate taken up in sufficient buffer to bring the volume to one-half the original globulin volume. **Preparation of muscle tissue:** The muscle tissue studied was derived mainly from human biopsy material. In addition, muscle from cat, rabbit, rat and mouse was used. The majority of muscle preparations were made by gentle teasing of the fibers after washing in 50% glycerol for a period of time, from several hours to several days. Other preparations were frozen in isopentane chilled with liquid nitrogen to -150°C, and sectioned at 8-10 m μ in a cryostat at -18°C. Human biopsy material in which pathological changes were suspected was in some instances fixed in chilled 10% formalin and embedded in diglycol stearate for sectioning. All preparations were treated with fluorescent anti-human myosin rabbit globulin solution for 20 minutes. The excess of fluorescent antibody solution was washed away with 5-6 changes of cold buffered saline solution. The preparations were mounted in 25% glycerol and examined and photographed under the fluorescence microscope. Study of the preparations in ultraviolet light was supplemented by observations in phase contrast and polarized light. In addition, some sections, particularly the pathological material, were stained by routine histological methods. As controls for the specificity of the fluorescent antibody staining, duplicate preparations were treated with fluorescent normal globulin solution. Also as an additional control, muscle sections were treated

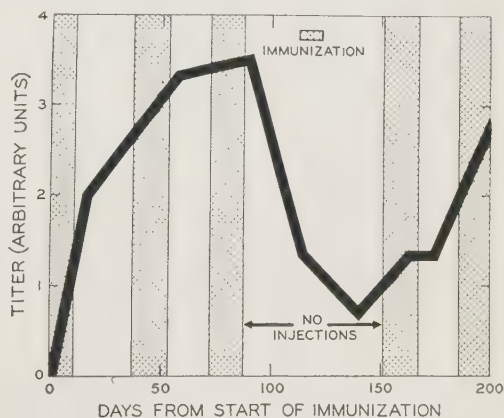


FIG. 2. Titer of antisera in relation to administration of myosin. Shaded areas—series of injections.

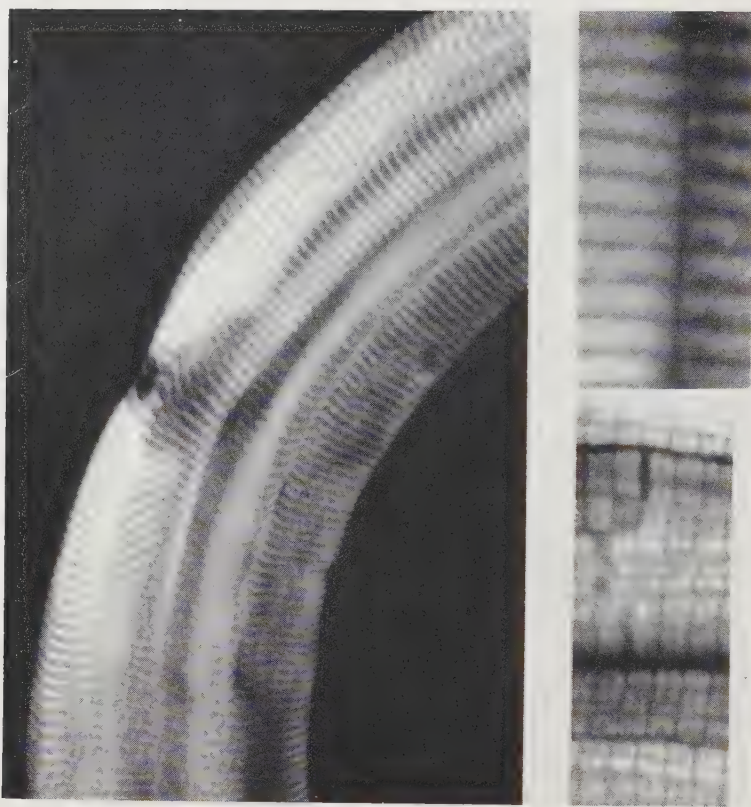


FIG. 3 (left). Human skeletal muscle. Teased preparation showing fluorescence of A bands; nuclei remain unstained. $\times 240$.

FIG. 4 (upper right). Human skeletal muscle. A band is clearly visible. $\times 1250$.

FIG. 5 (lower right). Human heart muscle. Intercalated discs remain unstained. $\times 1250$.

with a preparation of fluorescent antistreptococcal hyaluronidase globulin which was available from other studies(6).

Results. In the preparations stained by incubation with fluorescent normal globulin and anti-hyaluronidase globulin the muscle fibers showed very faint, diffuse, non-specific fluorescence.

Normal human skeletal muscle and muscle of cat, rabbit, mouse and rat stained with labelled anti-human myosin antibody revealed brightly fluorescent cross bands, which were identified in phase contrast and in polarized light to be A bands (Fig. 3). The sarcolemmal nuclei always remained unstained; on the other hand, the sheaths occasionally showed some fluorescence, presumably due to non-specific absorption or from failure to wash away the excess of the conjugate. The intensity of the fluorescence was strongest in

the teased preparations (Fig. 4). In the heart muscle, obtained from an autopsy case, the intercalated discs remained unstained (Fig. 5).

In muscle prepared by freezing and cutting in the cryostat most of the areas failed to show the characteristic striated pattern due both to the angle of cutting as well as distortion and fragmentation of myofibrils. In some areas, however, which were cut transversely, the myofibrils were sharply outlined as brightly fluorescent dots separated by non-staining interfibrillar spaces (Fig. 6).

Preparations fixed in formalin and embedded in diglycol stearate exhibited less intense fluorescence. Nevertheless, these preparations were valuable in the observations on pathological material because of the preservation of structural relationship between the fibers and interstitial elements.

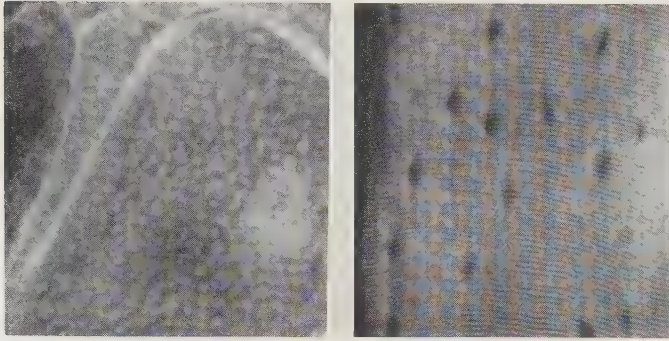


FIG. 6 (left). Cross-section of the rat muscle cut in the cryostat. Some of the myofibrils are brightly fluorescent. Fluorescence of the sarcolemmal sheath probably due to the failure of removing excess of the conjugate. $\times 400$.

FIG. 7 (right). Human muscle obtained by biopsy in a case with myotonic dystrophy. Muscle fibers show slight decrease in size with a relative increase in number of nuclei. Pattern of cross-striation is well preserved. $\times 240$.

Muscle tissue obtained from several patients afflicted with neuromuscular disorders presented histologically a variety of pathological changes. The muscle fibers in which pathological change was confined to an increase in the number of nuclei showed in ultraviolet light the preservation of the striated pattern with numerous oval or spherical unstained spaces, corresponding to the nuclei (Fig. 7). It was also noted that in some instances fibers undergoing severe degeneration were brightly fluorescent, and at the same time showed a complete loss of striated pattern, while the numerous condensed nuclei remained unstained (Fig. 8). Occasionally, single, small fluorescent bodies containing unstained spherical or oval spaces corresponding in size to the nuclei, were observed adjacent to the degenerating fibers (Fig. 9). Whether these structures were macrophages or remnants of the muscle fibers was not determined.

Discussion. The observations reported above demonstrated the specific staining of A bands in the striated muscle by antihuman myosin fluorescent antibody. The implication from this, however, that localization of myosin is confined to A bands must be considered with some reservations. It is well to keep in mind the difficulties associated with the estimation of the purity of myosin preparations. The methods employed for the estimation of purity (ultra-centrifugation, viscosimetry) are sensitive enough to exclude the presence of more than a few per cent ac-

tin, but are relatively insensitive to other impurities which may be associated with myosin. It is possible that even relatively small amounts of impurities in the preparation used for immunization may affect antibody re-

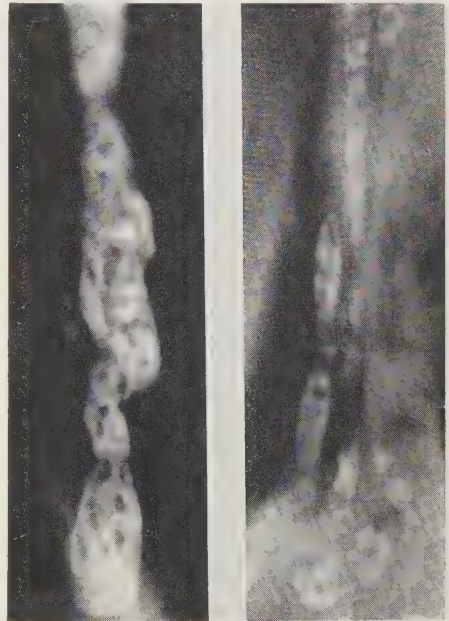


FIG. 8 (left). A severely atrophic fiber from the same case. It is brightly fluorescent with loss of striated pattern and with numerous unstained nuclei. $\times 400$.

FIG. 9 (right). Human muscle from another case of myotonic dystrophy. Between 2 well-preserved fibers lies a long thin atrophic muscle fiber with fluorescent material. In addition there are several scattered smaller oval bodies which may be macrophages containing fluorescent material. $\times 400$.

sponse to these substances. The occasional staining of sarcolemmal sheaths or Z band thus may be due to the presence of these additional antibodies other than myosin. Also, a theoretical possibility must be kept in mind that antigenic groups of myosin in certain sectors of the myofibril may be bound chemically to other substances and remain unreactive with antibody. This possibility prevents the absolute proof that myosin is absent in unstained segments such as the I band.

The presence of antibodies to myosin proper is demonstrated by the specific staining of myofibrils on cross sections, as these structures are known to be mainly composed of myosin. The finding that the nitrogen content of the washed precipitates agrees with theoretical figures indicates that the fluorescent globulin preparations mostly contained antibodies to myosin proper. It may be mentioned here that the fluorescence of the A band was already observed by Finck *et al.* (3).

The observation that anti-human myosin antibody reacted with muscle of other species raises the problem of antigenicity of myosin. Kesztyüs *et al.* (1) have found that of the 2 muscle proteins they studied actin was iso-antigenic and not species specific, myosin was not iso-antigenic. The observations reported here indicate that myosin is not species specific, since antibody to human myosin reacted with myosin prepared from the muscle of other animals.

The few observations on human muscle tissue undergoing degenerative changes should be regarded as preliminary ones. More experience in interpretation of fluorescent preparations must be obtained from the study of a larger amount of material. The quality of preparations which would preserve structural

relationships should be improved and it also seems desirable to extend these studies to include other muscle proteins. It is interesting, however, at this stage to note that material staining specifically with anti-myosin fluorescent antibody may remain condensed in markedly atrophic fibers or may eventually be transferred to macrophages.

Summary. 1. Fluorescent antibody technique has been used for study of localization of myosin in normal and diseased skeletal muscle. 2. A method for assay of antibody to human myosin has been described. 3. Specific staining with fluorescent antibody was obtained in A bands of the striated muscle. 4. According to observations on staining with fluorescent antibody and to precipitin reactions myosin has to be considered as not species specific. 5. Preliminary observations on diseased muscle stained with fluorescent antibody revealed the preservation of striated pattern at the time of early pathology changes, and the presence of material reacting with the antibody to human myosin in severely degenerated fibers and in structures resembling macrophages.

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Action of Antidiuretic Hormone in Potassium-Depleted Rats; Relation to Aldosteronism. (23669)

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The syndrome of primary aldosteronism, usually caused by a functioning adrenocortical tumor ("aldosteroma"(1) or "Conn's tumor") is characterized by intermittent tetany, paresthesias, periodic muscle weakness, polyuria, polydipsia and hypertension, but *no edema*(2). The significant metabolic findings are hypokalemic alkalosis, hyperaldosteronuria and hyposthenuria which is unresponsive to antidiuretic hormone (ADH)(1,2,3).

Aldosterone causes sodium retention(4,5) and would therefore be expected to cause concomitant water retention, as do the other adrenal steroids. The fact that primary aldosteronism is associated with polyuria instead of edema calls for an explanation. The clinical observation that the hyposthenuria in primary aldosteronism is unresponsive to ADH and to sodium chloride suggests that the polyuria and water loss are due to ineffectiveness of endogenous ADH, just as in nephrogenic diabetes insipidus. In the later condition, the distal tubules are unresponsive to ADH due to a hereditary abnormality. One wonders, then, if aldosterone "blocks" the action of ADH on the otherwise normal tubule.

In secondary aldosteronism observed in cardiac failure, hepatic failure, nephrosis and eclampsia, high aldosterone levels are not associated with polyuria, but with edema. It therefore appears unlikely that aldosterone "blocks" or counteracts ADH action in the renal tubules.

Since severe hypokalemic alkalosis is associated with primary and not with secondary aldosteronism, it was thought of interest to determine whether potassium depletion would influence the action of ADH.

Two recent observations point in this direction. Hollander, *et al.*(5) have shown that ADH fails to produce urine concentration in potassium-depleted rats, the intensity of the hyposthenuria being related to the degree of potassium depletion. Five adult patients of Relman and Schwartz(6) with severe diarrhea

producing severe potassium depletion demonstrated a vasopressin (Pitressin) resistant hyposthenuria. Urine volume and water retention were not mentioned in either paper.

The present experiment is designed to measure the amount of water retention induced by ADH in rats depleted of potassium.

Materials and methods. Thirteen of 25 male rats of the Wistar strain weighing between 106 g and 170 g were fed a diet deficient in potassium for 100 days, while 12 controls received a stock diet (Purina laboratory chow) restricted only in quantity to keep their combined weights down to that of the experimental group. The potassium deficient diet(7,8) for the first 28 days was raw rice, 45%; dextrose, 28%; casein, 20%; cod liver oil, 3%; yeast, 2%; ammonium chloride, 1%; sodium chloride, 1%; thiamin, 75 mg/kg of food and distilled water *ad libitum*. When signs of severe hypokalemia (anorexia and weight loss) failed to appear, the diet was changed to dextrose, 35%; non-nutritive cellulose (Alphacel), 27%; casein, 25%; yeast, 4%; cod liver oil, 4%; sodium bicarbonate, 3%; sodium chloride, 1%; ammonium chloride, 1%; and distilled water *ad libitum*. No steroids or other drugs were used.

Potassium determinations were not performed, but the following presumptive evidence of potassium depletion in the test rats is strong: the diet was virtually potassium-free; only the test rats became lethargic; anorexia, as evidenced by flattening then dipping of the weight curve, has been the experience of other workers(5,7,8,9,10).

On the 12th, 19th, 27th, 31st, 35th, 49th, 61st and 100th days, food and water were withdrawn and 2 hours later all rats were hydrated by an injection of 10 ml of distilled water intraperitoneally. Both the rats on the stock diet, and those on the potassium deficient diet were divided into 2 equal groups so that each of 8 experiments were carried out with 4 groups. One stock diet group and one

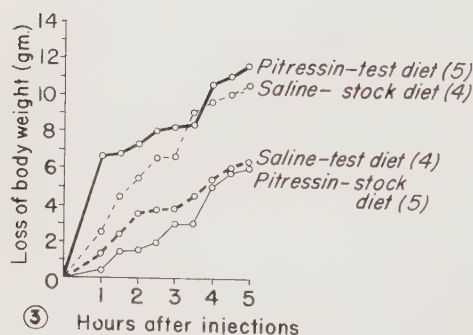
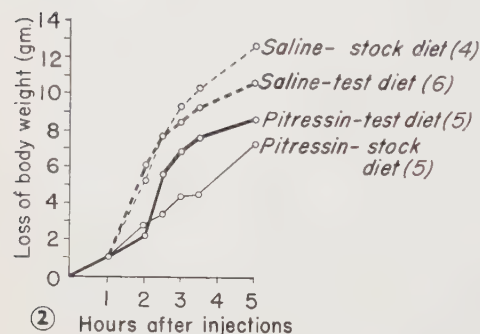
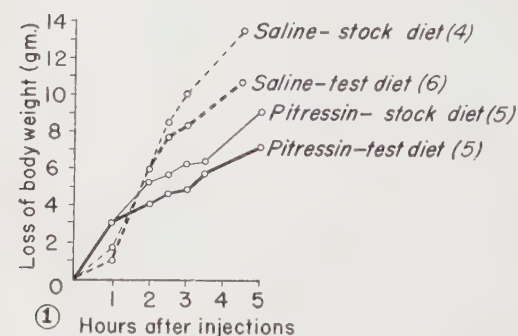


FIG. 1. Weight loss of hydrated rats over a 5-hr period on the 31st day of diet. One potassium deficient and 1 control group injected with 2 IU Pitressin, 1 potassium deficient and 1 control group injected with vehicle (saline) only. Numbers in brackets indicate the number of rats in each group. The variation in weight losses within each group of 4-6 rats at 3 hr, the point at which the curves were analyzed for significance, is given by a standard deviation of ± 1.75 g/group.

FIG. 2. Weight loss of hydrated rats over a 5-hr period on the 35th day of diet. One potassium deficient and 1 control group injected with 2 IU Pitressin, 1 potassium deficient and 1 control group injected with vehicle (saline) only. Numbers in brackets indicate number of rats in each group. Variation in weight losses within each group of 4-6

test diet group were injected subcutaneously with 2 IU of aqueous vasopressin (Pitressin, Parke Davis) in 0.5 ml of isotonic saline solution. The rats of the other 2 groups, one on stock diet and one on potassium deficient diet, served as controls and received 0.5 ml of isotonic saline subcutaneously.

All rats were weighed to the nearest gram immediately after being injected, and then weighed again at appropriate intervals. All rats were squeezed abdominally before each weighing to stimulate micturition. Feces were not weighed since the weight of even relatively large amounts is insignificant. In consecutive experiments control groups (saline only) and experimental groups (Pitressin) were rotated, the control groups of one experiment serving as experimental groups in the following.

Results. On about day 33, the weight curves began to flatten and on the 90th day they began to descend. The test rats had become progressively more lethargic beginning about the 50th day. The change in the rates of excretion of the 10 g water load became progressively more significant as the length of time on the test diet increased. Since the results of the first 3 injections were not significantly different from those of the 4th, and the 6th and 7th produced the same results as the 5th, only the results of the 4th, 5th and 8th experiments will be reported in detail. The control groups automatically cancel variations of body weight due to humidity, temperature, etc.

Fig. 1 shows average water loss over a period of 5 hours on the 31st day of the diet (3rd day of the improved diet), just before the test animals began to manifest signs of potassium depletion. The mildly potassium-depleted animals seemed to excrete their water load slower than the controls, but the

rats at 3 hr is given by a standard deviation of ± 1.33 g/group.

FIG. 3. Weight loss of hydrated rats over a 5-hr period on the 100th day of diet. One potassium-deficient and 1 control group injected with 2 IU Pitressin, 1 potassium-deficient and 1 control group injected with vehicle (saline) only. Numbers in brackets indicate number of rats in each group. Variation in weight losses within each group of 4-5 rats at 3 hr is given by a standard deviation of ± 2.46 g/group.

difference was not statistically significant. Pitressin slowed the water excretion in both groups to a highly significant degree ($P < .001$)* after a small initial diuresis.

Fig. 2 represents results of the injections given on the 35th day, just after the test rats began to show signs of potassium depletion. There was significant interaction ($P < .05$); *i.e.*, the injection effect was not independent of the diet effect. The effect of Pitressin on water retention in the normal animals was again highly significant ($P < .001$); in the moderately potassium-depleted rats Pitressin failed to induce a significant amount of water retention ($P > .05$). In the 2 groups given Pitressin, failure of the moderately depleted rats to retain as much water as the controls was probably significant ($P < .05$).

Fig. 3 shows how severely potassium-depleted rats (showing increasing lethargy and progressive weight loss) handle a water load with and without Pitressin. Again, there was significant interaction ($P < .01$). The severely depleted rats given a dose of isotonic saline seemed unable to excrete the water as fast as those on the control diet, but the difference was not statistically significant. Whereas Pitressin had previously produced water retention, now it not only failed to produce water retention in the depleted animals ($P < .05$), but actually enhanced its excretion in the first hour after injection. Furthermore, of the 2 groups given Pitressin, the depleted rats excreted their water significantly faster ($P < .01$).

Discussion. Experiments here presented show that rats fed a potassium-deficient diet become progressively less responsive to the antidiuretic action of Pitressin; of the severely potassium-depleted animals, the Pitressin-treated animals even lost water more rapidly than their controls. These results, as well as the work of Hollander, *et al.* (5), suggest that severe potassium depletion "blocks" the action of ADH. Welt (12) had previously suggested this hypothesis. The mechanism by which this "block" is brought about is, at

present, unknown. It is of interest that the effects of potassium depletion on ADH action seem to be biphasic inasmuch as mild potassium depletion appears to enhance the antidiuretic effect of ADH, whereas in severe potassium depletion ADH seems to induce diuresis.

These results may have a bearing on the differences in water balance in primary and secondary aldosteronism. The fact that ADH is ineffective in the presence of potassium depletion may explain the absence of edema in primary aldosteronism. In secondary aldosteronism, severe potassium depletion is not observed and endogenous ADH would be expected to be fully effective; if mild potassium loss would occasionally occur, the effect of ADH might even be potentiated. The polyuria associated with high levels of aldosterone seen in primary aldosteronism would then be analogous to nephrogenic diabetes insipidus, since endogenous ADH is ineffective in both; in the case of nephrogenic diabetes insipidus, polyuria is due to a primary defect in the kidney tubules, while in primary aldosteronism it is probably due to the ineffectiveness of endogenous ADH resulting from severe potassium depletion.

The results reported here may possibly have therapeutic implications. It is conceivable that in cases of secondary aldosteronism, especially nephrosis and cirrhosis, the production of hypokalemia with adrenocortical steroids, with or without added ADH, might induce a prompt diuresis, as seen in the test rats.

Summary and conclusions. Rats were depleted of potassium and half the group was injected with ADH when mildly depleted, moderately depleted and severely depleted; simultaneously, half the rats on stock diet were given ADH. The antidiuretic action of ADH was enhanced in the presence of mild potassium depletion; in moderate potassium depletion the antidiuretic effect of ADH was diminished; in severe potassium depletion ADH induced prompt polyuria. These results suggest that the lack of edema, and the presence of polyuria in primary aldosteronism are due to ineffectiveness or to blocking of endogenous ADH, not as a result of a di-

* All data were tested at the 3 hour point after injection. Results were analyzed statistically by the method of factorial arrangements (11).

rect antagonistic action of aldosterone, but as a result of the potassium depletion.

The author is indebted to Dr. Karl E. Paschkis for his interest and invaluable advice, and to Dr. Hyman Menduke for the statistical analysis of data.

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NaCl Appetite of Adrenalectomized Rats.* (23670)

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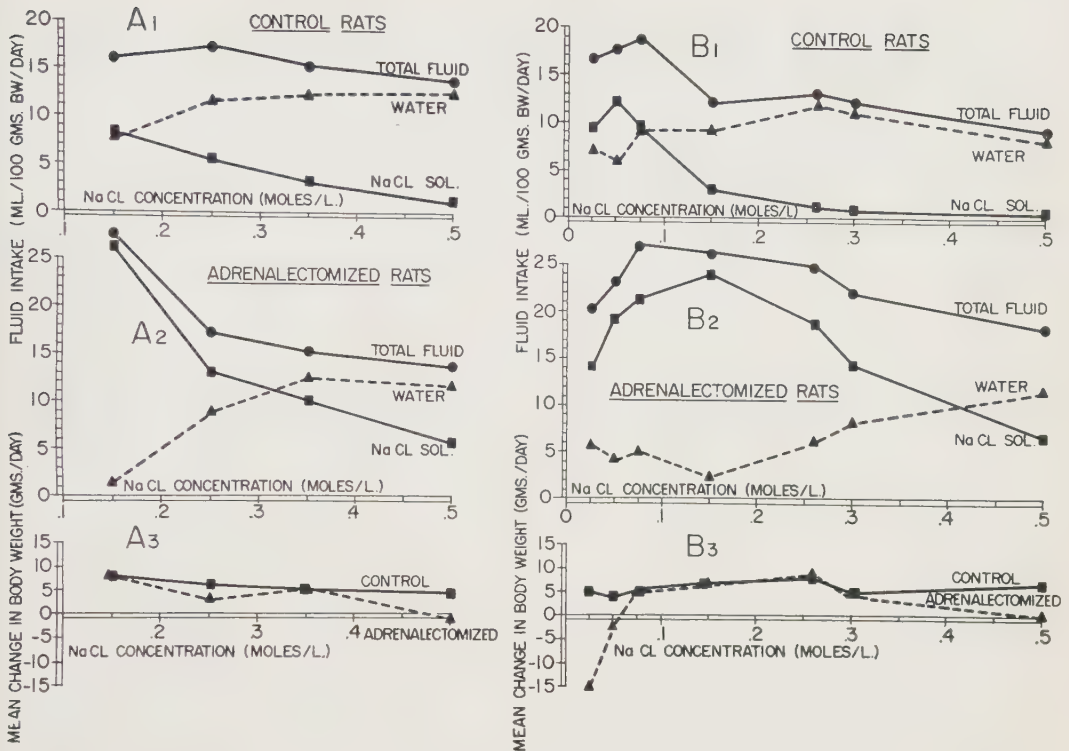
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One of the striking behavioral characteristics of adrenalectomized rats is their spontaneous NaCl appetite when given choice between water and .15 M NaCl solution as drinking fluids(1,2). This characteristic has been used by some investigators to assess completeness of adrenalectomy(3,4). It was therefore deemed worthwhile to determine whether the NaCl appetite of adrenalectomized rats was concentration dependent; that is, whether the rats preferred NaCl solution to water at some but not all concentrations of the former. It was also the object of this study to determine the NaCl concentration at which adrenalectomized rats ingested most salt solution. Knowledge of this particular concentration would be especially useful in experiments in which completeness of adrenalectomy is to be assessed, since it would assure that maximal differences could be obtained between intakes of normal and adrenalectomized rats.

Methods. Two separate experiments were performed. The materials and methods, similar for both experiments, are described below. Male rats of the Holtzman strain were used.

All rats were kept in individual cages in a room maintained at $26 \pm 1^\circ\text{C}$ and illuminated from 8 a.m. to 6 p.m. The animals were allowed Purina Laboratory Chow *ad libitum*. Intakes of food were not measured. Each rat was given choice of 2 drinking fluids, distilled water and NaCl solution. The type of fluid containers used has been described (5). The positions of the 2 bottles were interchanged daily to avoid habit formation in the selection of the fluids. Fluid intakes were measured daily by weight difference and are expressed as ml/100 g body weight/day. Body weights of the rats were also measured daily. **Exp. 1.** Five adrenalectomized and 5 control rats ranging in initial weight from 180 to 200 g were used. Adrenalectomy was performed one week prior to beginning any measurements, and all animals were maintained on .15 M NaCl solution and water until the experiment began. Each of 4 different concentrations of NaCl solution was offered, with distilled water as the second fluid choice, for 4 days. Chronologically the concentrations given were: .25, .35, .50 and .15 M. **Exp. 2.** Five adrenalectomized and 5 control rats ranging in initial weight from 220 to 240 g

* Supported by grant from American Heart Assn.



FIGS. 1A and 1B. Water, NaCl and total fluid intake of control (A_1 and B_1) and adrenalectomized (A_2 and B_2) rats in Exp. 1 and 2 respectively. Changes in body weight of both groups of rats in each experiment are shown in A_3 and B_3 .

were used in this study. They were not the same animals used in the first experiment. Adrenalectomy was performed one week prior to the beginning of the experiment. All rats were given choice between water and each of 7 different concentrations of NaCl solution. This experiment differed from the first in that the rats were offered choice between a test concentration of NaCl and distilled water for one day and then were given choice between .15 M NaCl solution and water for 2 days. Choice was then given between the second test concentration and water for one day followed by 2 more days with choice between .15 M NaCl solution and water. This pattern was followed throughout the experiment to prevent the rapid decreases in body weight of adrenalectomized rats accompanying low NaCl intake. Chronologically the test concentrations of NaCl given were: .15, .075, .050, .025, .26, .30 and .50 M. At the end of each experiment, completeness of adrenalectomy was assessed by removal of the salt so-

lution only. Rats from whom adrenals have been completely removed do not survive longer than 15 days without supplementary NaCl(6). Completeness of adrenalectomy was also assessed by gross inspection for adrenal tissue at autopsy. Statistical analysis of the differences between means was performed by the use of the "t" test for the 5% confidence limit(7).

Results. The results of Exp. 1 are shown in Fig. 1A. It may be observed that control rats ingested similar ($P > .05$) volumes of salt solution and water when the test concentration was .15 M. When the NaCl concentration increased to .250 M, the volume of NaCl solution ingested decreased significantly ($P < .05$) below the simultaneous water intake (Fig. 1A₁). Total fluid intake remained relatively constant but decreased when the highest concentration was given (.50 M). Adrenalectomized rats ingested over 3 times as much .15 M NaCl solution as control rats (Fig. 1A₂). As the concentration of the NaCl

TABLE I. Mean Fluid Sodium Intake of Adrenalectomized and Normal Rats at Each NaCl Concentration Offered.

| | NaCl concentration | | | | | | | |
|---------|--|--------|--------|-------|-------|-------|-------|-------|
| | .025 M | .050 M | .075 M | .15 M | .25 M | .30 M | .35 M | .50 M |
| Exp. 1 | Mean fluid Na intake (meq/100 g body wt/day) | | | | | | | |
| Adx. | | | | 3.96 | 3.26 | | 3.54 | 3.96 |
| Control | | | | 1.25 | 1.38 | | 1.10 | .57 |
| Exp. 2 | | | | | | | | |
| Adx. | .36 | .96 | 1.63 | 2.71 | 4.86 | 4.31 | | 3.36 |
| Control | .24 | .61 | .72 | .56 | .29 | .30 | | .38 |

solution increased, the volume ingested decreased markedly. When .35 M, or higher, NaCl solution was given, the adrenalectomized rats ingested significantly less salt solution than water. The total fluid volume ingested by adrenalectomized rats also decreased as the concentration increased. Control rats gained in body weight throughout the experiment while adrenalectomized rats showed a decrease in body weight only when .50 M NaCl solution was given (Fig. 1A₃).

The results of Exp. 2 are qualitatively similar to those of Exp. 1 and are shown in Fig. 1B. With the wider range of NaCl concentrations offered, control rats drank most NaCl solution when the concentration was .050 M. At all concentrations above .075 M control rats ingested more water than salt solution (Fig. 1B₁). Total fluid intake was highest when hypotonic concentrations were given (.025, .050, .075 M); relatively constant when .15, .26 and .30 M were given and lowest when .50 M was given. In the case of adrenalectomized rats, the largest volume of NaCl solution was ingested when .15 M NaCl solution was given. At concentrations higher than this, less and less NaCl solution was ingested. When .50 M NaCl solution was offered, significantly more water than salt solution was ingested. Total fluid intake of adrenalectomized rats also varied, decreasing as NaCl concentration increased.

Control rats gained weight throughout the experiment without regard to the concentration of the salt choice offered (Fig. 1B₃). Adrenalectomized rats, on the other hand, lost weight when the 2 lowest concentrations were offered and failed to gain weight when the highest concentration was offered. The greatest weight gain occurred when .25 M NaCl

solution was given.

Table I shows that the sodium taken in (meq/100 g B.W./day) by both adrenalectomized and control rats with their drinking fluids was relatively constant in the first experiment. The exception to this was the control group which decreased significantly ($P < .05$) its sodium intake below any of the previous levels when .50 M NaCl solution was given.

In the case of the second experiment in which a wider range of concentrations was offered, sodium intake was more variable in both groups of rats. The sodium intakes of adrenalectomized rats given .15, .25, .30 or .50 M NaCl solution did not differ from each other statistically ($P > .05$). However, sodium intakes of these rats given .025, .050 and .075 M NaCl solutions were significantly different from each of the higher concentrations. The variability in sodium intake from rat to rat was quite large. The sodium intakes of control rats were not different, one from the other, throughout the experiment. It is worth noting, however, that both groups of adrenalectomized rats ingested 2 to 17 times as much sodium by way of their drinking fluid as their respective controls.

When NaCl was removed from the cages of all rats at the end of both experiments, all adrenalectomized rats died within 10 days (mean 5.8; range 3 to 10 days). Autopsy of these rats did not yield adrenal tissue. It was therefore assumed that all the rats were completely adrenalectomized.

Discussion. The NaCl appetite of adrenalectomized rats has been shown to be concentration dependent in that less NaCl solution than water was ingested if the concentration of the NaCl solution was .35 M or greater.

The largest volume of NaCl solution was ingested when adrenalectomized rats were given .15 M. At this time the intake of NaCl solution represented 90 to 95% of the total fluid intake. A very large volume of .075 M NaCl solution was also ingested by adrenalectomized rats and consisted of 80% of the total fluid intake. On the other hand, control rats generally preferred water to NaCl solution except in low concentrations (.025 to .10 M). On the basis of this study, it would seem that .15 M NaCl solution is the best concentration to use for assessment of completeness of adrenalectomy. At this concentration the difference between intakes of salt solution of adrenalectomized and normal rats was maximal (Fig. 1).

The total fluid intakes of both groups of rats was greatest when hypotonic concentrations of NaCl solution were given (Fig. 1A and 1B). Conversely, the lowest total fluid intakes were observed when the highest concentration of NaCl was given (.50 M). It is difficult to explain from the data presented why both groups should take in more fluid in the hypotonic concentration range and less in the hypertonic concentration range.

In a multiple choice experiment, Young and Chaplin(8) gave normal and adrenalectomized rats simultaneous choice of 8 different NaCl solutions and reported that both groups of rats ingested most of their NaCl solution from the bottle containing a .12 M solution. The results of the single choice experiment reported here for adrenalectomized rats agrees with that reported by Young and Chaplin in that these rats ingested most salt solution when .15 M concentration was given. In the case of control rats, however, the results are different. The maximal volume of salt solution was ingested when .050 M was given. The different results may be due to the difference in experimental procedures; *i.e.* multiple *vs.* single choice.

Table I indicates that the pattern of the curves of both groups of rats in Fig. 1 cannot necessarily be attributed to attempts to maintain fluid sodium intake constant. When a wide range of NaCl concentrations was offered in Exp. 2, the sodium taken in by adrenalectomized rats did not remain constant. It is

interesting that these rats should ingest nearly half as much water as NaCl solution when the concentrations lay below .075 M. At these concentrations one might expect the adrenalectomized rats to ingest salt solution to the exclusion of water, especially since adrenalectomized rats are known to have a preference ("taste") threshold several fold lower than that of normal rats which is known to lie between .008 and .016 M(9,10).

From the data presented, it cannot be stated accurately that the amount of sodium ingested with food remained constant. Under similar experimental conditions, however, it has been observed that the food intake, and therefore the food sodium intake, remained relatively constant (1.2 to 1.3 meq/100 g B.W./day)(10). It seems unlikely that the food intake of adrenalectomized rats could have varied to the extent necessary to maintain a constant sodium intake. Further study will be necessary to determine the factors influencing the pattern of the curves in Fig. 1.

Summary. In single-choice experiments adrenalectomized and normal rats were given choice between distilled water and each of a series of NaCl solutions. The NaCl appetite of adrenalectomized rats was shown to be concentration dependent. Adrenalectomized rats preferred NaCl solutions to water until the concentration of the salt solution was .35 M or greater. At these higher concentrations the rats preferred water to salt solution. Adrenalectomized rats ingested most NaCl solution (ml/100 g B.W./day) when the concentration was .15 M. These rats either lost weight or ceased to gain weight when low (.025 to .050 M) and high (.50 M) NaCl concentrations were given. Body weight of control rats was not influenced by the concentration of NaCl solution offered. Control rats preferred NaCl solution to water at all concentrations below .075 M. At concentrations greater than .15 M, the rats drank more water than NaCl solution. The greatest volume of NaCl solution (ml/100 g B. W./day) was ingested by control rats when a concentration of .05 M was given. The maximal difference between intakes of NaCl solution of control and adrenalectomized rats occurred when .15 M was given. Hence, this concentration

would appear to be best fitted to test completeness of adrenalectomy.

It is a pleasure to acknowledge the technical assistance of Mr. O. Galindo and Mr. L. Collier. I thank the Department of Medical Illustrations for drawing and photographing the Figure.

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Response of Individual Muscles of the Guinea Pig to "Large" Doses of Testosterone Propionate.* (23671)

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Administration of testosterone and several other androgens at various doses by multiple pellet implantation restored the individual muscles of the castrated male guinea pig only to normal(1). An attempt was made in this study to produce a response greater than normal by injection of "large" doses of testosterone propionate for a "long" period of time.

Procedure. Guinea pigs from the Zeimet Bio-farms were maintained in individual cages in an air conditioned room. They were fed Rockland Guinea Pig diet and supplements of 30 g of carrots and 25 mg of Vit. C twice per week. They were castrated at 68 days of age when they weighed 549 to 698 g. Injection of testosterone propionate† was begun on the day of castration and continued daily except Sunday for 65 days.

At autopsy the internal organs were weighed. The carcasses were sealed in plastic bags and kept frozen until the muscles were dissected.

Results. The castrated animals showed the expected(2,3) smaller gain in body (cf

carcass) weight with the accompanying decreases characteristic of the respective muscles (Fig. 1). The 5 and 10 mg/day doses of testosterone propionate maintained all of the muscles except the temporal and digastric at or within the normal level. No significant differences between the 2 dose levels were detected. The 25 mg/day of testosterone propionate not only maintained many of the muscles at the normal level but also increased several above normal. These increases were less than 20% and occasionally as high as 30% (Fig. 1).

The seminal vesicles and prostates as well as the retractor penis did not show an excessive enlargement under the stimulation of the very high doses of testosterone propionate (Fig. 1).

The internal organs showed no remarkable changes in weight(1,2,3). The heart weight was slightly decreased after castration and maintained by the androgen. The thymus showed no change after castration but a slight decrease after androgen treatment. All of the other organs showed no changes.

Discussion. The guinea pig apparently possesses some mechanism(s) to protect it from excessive dosage of androgens. This phenomenon is not specific for any particular

* This investigation was supported by research grants from the Nat. Inst. of Arthritis and Metabolic Diseases, Public Health Service.

† The androgen was provided as perandren propionate by Ciba Pharmaceutical Products.

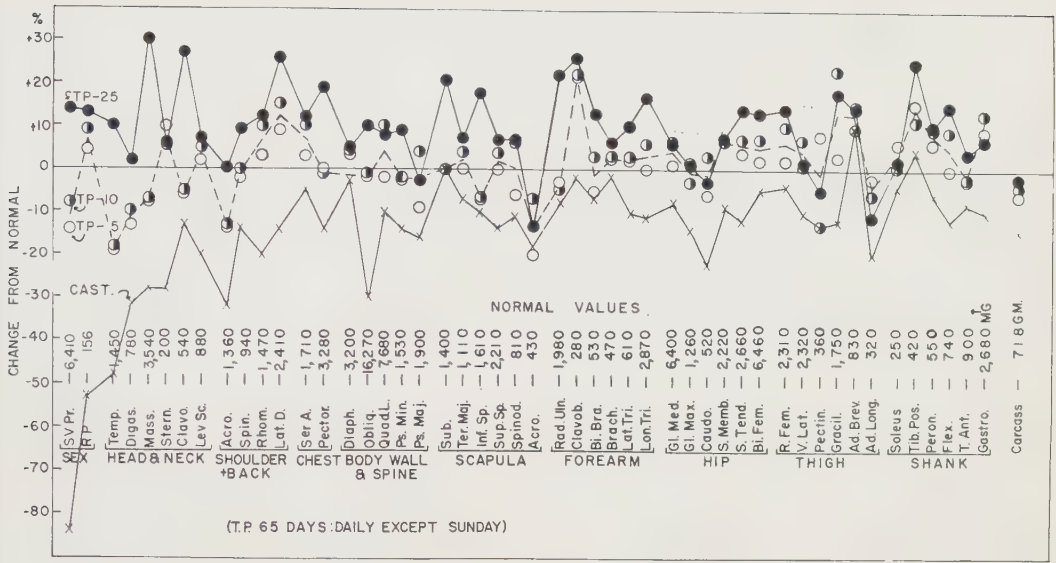


FIG. 1. Response of individual muscles of castrated male guinea pig to "large" doses of testosterone propionate. The guinea pigs were castrated and injections initiated at 68 days of age (549 to 698 g body wt).

tissue. None of the internal organs as well as the muscles and accessory sex organs showed an excessive response to the high doses of testosterone propionate. The guinea pig, therefore, differs from the rat(4), mouse(5) and hamster(6) which show excessive responses in the seminal vesicles and prostates to relatively high doses of androgens yet small relative to those administered to the guinea pig. Indeed the doses used in this study have proven to be effective growth stimulators in much larger species *e.g.* dog(7) and man(8).

It is of interest that the guinea pig in contrast to the rat and mouse does not lose weight (muscle) or decrease the size of the thymus or spleen when administered high doses of cortisone(9).

Summary. Male guinea pigs were castrated at 68 days of age and injected daily for 65 days with 5, 10 or 25 mg/day of testosterone propionate. The 2 smaller doses maintained the rate of growth of the body and that of 48 individual muscles with the exception of the

temporal and digastric. The highest dose not only maintained the rate of growth of the muscles but increased many slightly above normal.

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Production of Unesterified Fatty Acids from Isolated Rat Adipose Tissue Incubated *in vitro*. (23672)

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Recent studies of the concentration of unesterified fatty acid (UFA) in the circulating plasma of intact human subjects have given evidence that UFA is derived from adipose tissue, and that the amount in circulation and the rate of its production from adipose tissue vary in response to the metabolic requirements of the organism(1,2,3). The administration of carbohydrate, insulin, or both, to fasting subjects was shown to be a potent influence leading to a reduction of circulating UFA concentrations. Administration of epinephrine or fasting led to marked increases. The present study was undertaken to discover if corresponding effects could be demonstrated using isolated adipose tissue fragments incubated *in vitro*, since such a simplified system might lend itself to the demonstration of the role of the various factors concerned with the regulation of the liberation of UFA from depot fat.

Materials and methods. Adipose tissue for study was taken from the epididymal fat bodies of young male rats weighing 150 to 250 g; the animals were anesthetized with intravenous nembutal and the tissue removed prior to sacrifice. This tissue occurs in thin leaves and does not require slicing. The tissues were rinsed in a 1% solution of bovine serum albumin (Armour Fraction V) in saline, and stored briefly in this medium until all rats of a given group had been sacrificed. The tissues were then transferred into 25 ml Erlenmeyer flasks each containing 5 ml incubating medium, and agitated gently at 37°C. The flasks were covered with aluminum foil to prevent evaporation of the medium. The wet weight of each tissue fragment was determined at the end of the experiment, and suitable aliquots of the incubation medium were analyzed for UFA content at the beginning and end of each incubation, using the method previously published(2). The period of incubation was 4 hours except where otherwise noted. Incubations were carried out in a

medium prepared by dissolving 5% by weight of bovine serum albumin in Krebs-Ringer phosphate buffer, and readjusting the pH to 7.3-7.4 with sodium hydroxide. Results of UFA analyses were calculated in terms of micromoles of UFA liberated per gram wet weight of tissue per hour. Whenever possible, one epididymal fat body from a rat served as a control for comparison with the behavior of the contralateral tissue under the influence of the agent being tested.

Results. The effect of nutritional state was tested by comparing UFA production rate from the tissues of rats fasted overnight with the behavior of tissues from rats maintained overnight without solid food, but with 10% glucose in their water. The tissues from the carbohydrate-fed rats produced significantly less UFA on incubation than did those from the fasted animals (*p* less than 0.01). Data from one representative experiment are given in Table I.

The effect of epinephrine added to the medium at a concentration of 0.01 μ g per ml of solution was tested on the tissues of both fasted and carbohydrate-fed rats. In these experiments, incubation times of one hour were sufficient to demonstrate acceleration of UFA release in the epinephrine-treated tissues. The data from one typical experiment are given in Table II. This experiment alone is significant on statistical analysis (*p* equals 0.05); with 3 other experiments yielding similar results one cannot doubt the effectiveness of epinephrine at this concentration. At the concentration of 0.1 μ g per ml of medium epinephrine showed a highly significant effect

TABLE I. UFA Production from Tissues of Fasted and Fed Rats.

| State | UFA production (μ M/g tissue/hr) | |
|--------------------|--|------|
| | Mean | S.D. |
| Fasted (4 tissues) | 6.4 | .75 |
| Fed (4 ") | -.2 | .38 |

TABLE II. Effect of Epinephrine (0.01 $\mu\text{g/ml}$) on UFA Production from Tissues of Fasted Rats.

| Treatment | Mean UFA production ($\mu\text{M/g}$ tissue/hr) |
|--|---|
| None (4 tissues) | 1.31 |
| Epinephrine (4 ") | 2.31 |
| Differences between outputs of treated tissues and contralateral controls: | |
| Mean 1.00; S.D. .62 | |

(p less than 0.01); whereas at a concentration of 0.001 μg per ml, the effect was questionable.

The effect of insulin was tested on the tissues of fasted rats incubated in media fortified by the addition of 1 mg glucose per ml. 0.01 unit of insulin per ml of medium caused a significant decrease in the UFA output of tissues so treated. In Table III are presented the data from one experiment demonstrating this effect; the results of this one experiment are highly significant (p less than 0.01). Repetitions of the same experiment yielded confirmatory results.

Discussion. The demonstration of liberation of UFA from adipose tissue fragments incubated *in vitro* serves to support the conclusion, derived from studies of intact fasting human subjects, that the source of the circulating UFA is adipose tissue. It must be noted, however, that if transport of fatty acids out of the depots as UFA is to account for the mobilization of stored fat to supply the caloric needs of the organism during periods of fasting, a rate of UFA release on the order of 100 micromoles per gram of tissue per hour would be necessary. (This calculation applies to

TABLE III. Effect of Insulin (0.01 unit/ml) on UFA Production from Tissues of Fasted Rats.

| Treatment | Mean UFA production ($\mu\text{M/g}$ tissue/hr) |
|---|---|
| None (4 tissues) | 1.58 |
| Insulin (4 ") | -1.03 |
| Differences between treated tissues and contralateral controls: | |
| Mean 2.61; S.D. .43 | |

rats of approximately 200 g weight, and employs unpublished data of Dr. Joseph Bragdon for the average total body fat and caloric requirement of Sprague-Dawley rats.) It is evident, therefore, that the observed metabolic activity of adipose tissue *in vitro* falls short of its postulated activity *in vivo*. Such impairment of activity is not surprising, since the incubated tissues are exposed to the medium only on their external surface, whereas *in vivo* the surface available for exchange of metabolites with plasma is much greater. In spite of this objection, it seems likely that this *in vitro* system constitutes a satisfactory model for the study of chemical and physiologic factors influencing UFA production.

The nutritional state of the donor animal, and 2 hormonal substances added *in vitro*, have been shown to influence UFA production in the model system in a manner compatible with their observed influences *in vivo*. The epinephrine effect has been demonstrated with hormone concentrations which are low enough to be similar to those effective *in vivo*; insulin has so far been tested only at a concentration 2 orders of magnitude higher than that effective *in vivo*. Nevertheless, the effectiveness of insulin in this simple system suggests that it operates in the intact organism by affecting the adipose tissue directly rather than through the mediation of some other physiologic system.

Summary. Adipose tissue from rats, incubated *in vitro* in a medium of known composition, was found to release unesterified fatty acids (UFA). The rate of evolution of UFA was increased by the addition of epinephrine to the medium or by fasting the donor animal. It was decreased by the addition of glucose and insulin to the medium, or by feeding the donor animal with carbohydrate.

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An Evaluation of Various Tissues in Culture for Isolation of Eastern Equine Encephalitis Virus.* (23673)

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In 1936, Cox found that suspended fragment cultures of embryonic chick tissue could be employed to detect eastern equine encephalomyelitis virus in dilutions which were inactive on intracerebral inoculation of mice and guinea pigs(1). The presence of virus in such cultures was demonstrable by intracerebral inoculation of the supernatant fluid into mice. It was subsequently noted that this agent would multiply in cultures of other tissues as well, and that such multiplication was associated with a cytopathogenic effect on the cells(2,3,4,5,6). Despite these observations, however, tissue cultures have not been commonly employed for diagnostic or epidemiologic studies of this disease. Instead, 3-week-old mice, suckling mice or 9-12-day-old embryonated hens' eggs are usually recommended for such purposes(7). The propagation of poliovirus in cultures of human tissue by Enders, Weller and Robbins(8) served to reemphasize the value of tissue culture for the study of viruses, and this method has subsequently been extensively developed and widely applied. Accordingly, when epizootics and epidemics of EEE occurred in Massachusetts during 1955 and 1956, it was decided to evaluate the efficacy of such cultures for the isolation of these agents by comparing them with embryonated hens' eggs and suckling mice. The results of this study are herein presented.

Materials. *Virus.* Specimens of human, horse, pheasant, pigeon and sparrow cerebral tissue, naturally infected with EEE virus, were supplied by the Department of Pathology, Children's Medical Center, Boston, or through the courtesy of Mrs. Joan Daniels, Virus Section, Division of Laboratories, Massachusetts Department of Public Health.

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[†] Holder of a Fellowship in Tuberculosis administered for The National Tuberculosis Association by The Nat. Acad. Sci., Nat. Research Council.

Four specimens of mouse brain artificially infected with this agent were also employed. *Aedes aegypti* mosquitoes infected with EEE strain AR-167 were kindly provided by Dr. Roy W. Chamberlain, Communicable Disease Center, Public Health Service, Montgomery, Ala. This virus, originally isolated from specimens of *Culiseta melanura* was inoculated as second mouse-brain and first chick-embryo-passage materials into half day-old chicks. The latter, during the stage of viremia, served as the source of infection for the *Aedes* mosquitoes. The identity of these agents in the different materials was confirmed by neutralization tests using specific antisera and cultures of chick embryonic tissue. The mosquitoes were preserved in the CO₂ icebox. The remaining specimens were either stored in the form of tissue fragments at -15°C or as supernatant fluids of 10 or 20% suspensions of the tissues in the CO₂ chest. Such fluids will hereafter be referred to as "viral suspensions" or simply "suspensions." *Tissue Cultures.* The following types of cultures were utilized during this study: stationary tube cultures of trypsinized monkey renal, human amniotic, human renal, HeLa, Detroit 6, embryonic chick, embryonic mouse, and newborn mouse brain cells, and roller tube fragment cultures of human embryonic skin and muscle and embryonic chick intestine. For HeLa and Detroit 6 cell cultures, Eagle's medium with 5% or 10% inactivated horse serum was used(9). The nutrient for newborn mouse brain tissue consisted of bovine amniotic fluid 45%, Hanks' balanced salt solution 40%, inactivated horse serum 10% and beef embryo extract 5%. This medium was adjusted to pH 7.2 with CO₂ immediately prior to use. For cultures of the remaining tissues, the bovine amniotic fluid medium was also employed, but the concentration of Hanks' balanced salt solution was increased to 45% at the expense of the horse serum constituent. In addition 0.1 ml of a 1% solution of soybean inhibitor

per 20 ml of media was added to the cultures of human embryonic skin and muscle. All media contained 100 units of penicillin and mycostatin and 100 micrograms of streptomycin per ml. The media were usually changed before inoculation of the virus and whenever the pH fell below 7.0. When cellular outgrowth was abundant, the inoculum was added and the cultures incubated at 35°-37°C in a rotating drum. The cultures were then examined at intervals for cytopathogenic changes during a minimum period of 7 days. Simplified methods for preparation of embryonic chick and embryonic mouse tissue cultures are presented in detail below. A method for growing newborn mouse brain cells developed by Dr. Simon Thiery in this laboratory, is also given. Cultures of the remaining tissues were prepared by generally accepted procedures(10).

i. *Embryonic chick tissue cultures.* 7- to 9-day-old chick embryos were washed with isotonic phosphate buffer solution and incubated with agitation (magnetic stirrer) for 1 hour at room temperature in 0.25% trypsin (Difco) solution adjusted to pH 7.8. The released cells were separated by centrifugation at 1000 rpm for 10 minutes, washed once and then resuspended in nutrient medium. In one experiment approximately 30 ml of a suspension containing 8 million cells per ml was obtained from 7 embryos which had been treated with 60 ml of trypsin. Cultures initiated with 600,000 cells suspended in 1 ml of medium were usually ready for use after 24 hours. When the media was changed at appropriate intervals the cultures remained suitable for inoculation during at least one month. Suspensions of chick embryo cells have yielded satisfactory cultures after storage at 4°C for periods up to 1 week, although formation of the cell sheet was somewhat delayed. ii. *Embryonic mouse tissue cultures.* 6 to 8 embryos obtained from a 12- to 15-day gravid Webster strain Swiss mouse were usually employed as the source of tissue. The cell suspension was prepared in a manner similar to that for the embryonic chick cultures. However, trypsinization was allowed to proceed at 37°C instead of at room tem-

perature. Tubes prepared with a 1 ml inoculum consisting of 600,000 to 800,000 cells were suitable for use after 5 to 7 days. iii. *Newborn mouse brain tissue cultures.* Ten ml of 0.25% trypsin solution adjusted to pH 7.5 were added to a brain mince prepared from 4 to 8 ether-anaesthetized newborn mice. After 1 to 2 minutes incubation at room temperature the trypsin was discarded, a fresh aliquot added and trypsinization allowed to proceed for 5 to 10 minutes with occasional shaking. The cells were removed by centrifugation in the cold at 300 rpm for 30 minutes, washed once and resuspended in nutrient medium at a concentration of 1 million cells per ml. Cultures prepared with 1 ml of this suspension per tube were ready for use in 4-5 days.

Procedures. The evaluation of the various procedures for the isolation of the virus was carried out in 3 ways. First, cultures of various tissues were tested for susceptibility to virus strains as indicated by the appearance of cytopathic changes. Secondly, the infectivity titers of suspensions of tissues infected with a human, equine, avian and arthropod strain were determined in suckling mice, embryonated hens' eggs, and in cultures of certain tissues. Thirdly, aliquots of the same suspensions were titrated in cultures of embryonic chick and embryonic mouse tissue, and in the case of the arthropod material in cultures of newborn mouse cerebral tissue. Experiments with a given suspension of virus were carried out simultaneously in each of the systems to be compared. Dilutions of the suspension were prepared immediately prior to use and held in the cold until added to the cultures.

Comparative susceptibility of tissue cultures to EEE virus strains. The inocula were prepared by grinding specimens of infected cerebral tissue with sufficient isotonic phosphate buffer solution or bovine amniotic fluid medium to give a 10% suspension. Penicillin and streptomycin in concentrations of 100 μ and 100 μ g per ml respectively were included. Each specimen was centrifuged at 2000 rpm for 10 minutes at room temperature. Cultures of various tissues, usually in duplicate, were

TABLE I. Susceptibility of Various Tissues in Culture to EEE Virus from Human, Equine, Avian and Murine Sources.*

| | Embryonic chick | Monkey kidney | Human amnion | Human kidney | Human embryonic skin & muscle | HeLa |
|-----------------------|-----------------|---------------|--------------|--------------|-------------------------------|------|
| No. specimens tested | 19 | 19 | 18 | 18 | 12 | 15 |
| No. positive | 19 | 15 | 12 | 0 | 10 | 0 |
| % positive | 100 | 79 | 67 | 0 | 83 | 0 |
| No. cultures employed | 36 | 36 | 35 | 35 | 23 | 25 |
| No. positive | 35 | 28 | 23 | 0 | 17 | 0 |
| % positive | 97 | 78 | 66 | 0 | 74 | 0 |

* Two or more strains of virus from each source were tested in each tissue.

inoculated with 0.1 ml amounts of the supernatant fluid and incubated in the roller drum.

Titration of representative strains in the suckling mouse, embryonated hens' egg and in tissue culture. Isotonic phosphate buffer solution with 2% inactivated horse serum and antibiotics was employed as the virus diluent. Ten percent suspensions were prepared as described above from specimens of infected human, equine and avian cerebral tissue. Five infected mosquitoes were ground in 5 ml of diluent to provide a virus suspension from an arthropod source. Each suspension was centrifuged in the cold at 5000 rpm for 1 hour and 10-fold serial dilutions were prepared from each supernatant fluid. Six to 16 Webster strain Swiss mice, 1 to 3 days of age, were inoculated intracerebrally with 0.02 ml and intraperitoneally with 0.03 ml of each dilution tested. Concurrently, the same quantity of virus (0.05 ml) diluted to 0.1 ml was inoculated into the yolk sac of each of five 9- to 12-day eggs and into each of 4 or 5 cultures of the tissues selected for testing. The mice were observed during a period of 2 weeks for failure to thrive, inactivity, vomiting, convulsions or death; the eggs were examined at intervals during 7 to 10 days for decreased activity, hemorrhage or death of the embryos. Tissue cultures were observed for a minimum of 7 days. Dead mice and embryos were stored intact at -15°C , and appropriate specimens were subsequently examined to confirm the presence of virus. This was done by inoculating cultures of embryonic chick tissue with 0.1 ml of the supernatant fluid from a 10% suspension of the heads or brains and noting any cytopathic effect.

Titration of the 4 representative strains in

cultures of embryonic chick, embryonic mouse, and newborn mouse brain tissue. The inocula were prepared as indicated in the above paragraph and consisted of 10-fold dilutions of the viral suspensions. Each of 3 to 5 tissue cultures was inoculated with 0.1 ml of each dilution.

Results. In the first part of this evaluation cultures of 6 different kinds of cells were examined for cytopathic changes following the addition of 10% suspensions of 19 specimens representing 15 strains of virus. These suspensions were prepared from infected cerebral tissue of 4 human beings, (7 specimens), 3 horses, 5 birds (2 pheasants, 2 pigeons, 1 sparrow) and 4 mice. Of the murine specimens, 3 represented 1st mouse brain passage of the virus from 2 human cases and the 4th consisted of the 141st mouse brain passage of a stock laboratory strain. The results are presented in Table I and indicate that of the tissues tested cultures of embryonic chick cells were the most sensitive for detection of this agent, regardless of the source of the inoculum. In these cells, moreover, the cellular destruction was marked and usually progressed rapidly to completion, although areas of epithelial growth, when present, remained unaffected by the virus. Cultures of monkey renal and human amnion cells were not only less sensitive but in these tissues, the cytopathic effect was frequently limited to specific areas at the periphery of the cell sheet and progressed slowly or not at all.

In certain instances cultures of these tissues appeared to develop a resistance to the agent as manifested by rapid regrowth of the cell sheet after the appearance of cytopathic changes. This phenomenon was also noted to

TABLE II. Infectivity Titers of Representative EEE Virus Strains in the Suckling Mouse, Embryonated Hens' Egg and Tissue Cultures.

| Test system | Virus source | | | | |
|------------------------|--------------|-------------|-----------------|-----|----------------|
| | Human brain | Horse brain | Pheasant brain* | | Mosquito pool* |
| Tissue cultures | | | | | |
| Embryonic chick | 7.2† | 3.5 | 7.5 | 6.8 | 3.7 |
| Monkey kidney | 6.8 | 2.5 | 6.6 | | 3.6 |
| Human amnion | 4.4 | <2.0‡ | 6.6 | | 3.8 |
| Embryonated hens' eggs | 5.3 | 3.4 | | 7.2 | 5.3 |
| Suckling mice | 7.8 | 3.6 | | 7.7 | 5.9 |

* Titrations of 2 samples prepared from the same specimen and tested at different times.

† Negative log ID₅₀/0.1 ml; endpoints calculated by the method of Reed and Muench.

‡ Lowest dilution tested.

a lesser extent in cultures of human embryonic skin and muscle. The nutrient media in several of these cultures were replaced often enough to preclude persistence of the virus originally introduced. Nevertheless, subculture and titration of the fluid from these cultures provided evidence that multiplication of the virus was continuing in the absence of a discernible cytopathic effect even after 3 months. Similar observations have been reported with this agent in cultures of rat normal and tumorous cells and in HeLa cells by Gey and his associates (3,5) and by Chambers with western equine encephalomyelitis virus (11).

Human renal cell cultures were found to be unsatisfactory for isolation of the virus, since no grossly detectable cytopathic effect was observed. When HeLa or Detroit 6 cells were employed a slight increase in granularity and occasional areas of cellular degeneration were noted with certain specimens of virus. These changes, however, were not unlike those occasionally found, though in lesser degree, in uninoculated control cultures and were therefore considered as probably non-specific. When aliquots of the fluid phase from 9 of the HeLa cell cultures were tested for the presence of virus by subinoculation into cultures of embryonic chick tissue, 5 proved to be positive. The fluids from 9 human renal cell cultures were similarly tested and virus was found in 3 of them. Thus, in certain of the cultures where no clear cytopathic changes were observed, proliferation of the agent probably occurred.

The occasional presence of uninjured epithelial cells in infected cultures of embryonic chick tissue suggested that these cells were resistant to the destructive effect of the virus. This observation was confirmed by adding large doses of the virus to cultures of embryonic chick intestine which yields an outgrowth that is primarily epithelial. The few non-epithelial elements present were quickly destroyed, but the epithelial cells remained apparently unaffected.

In the second part of the evaluation the relative susceptibilities of the suckling mouse, embryonated hens' egg and cultures of various tissues to 4 specimens consisting of infected human, equine, avian and arthropod tissue were defined by determining infectivity endpoints in each of these systems. For these experiments cultures of embryonic chick, human amnion and monkey renal cells were selected since in them the cytopathic effect is marked and they are readily available in many laboratories. The results are presented in Table II and indicate that the suckling mouse, following combined intracerebral and intraperitoneal inoculation, was the most sensitive in revealing the presence of the virus. This finding was constant with each of the 4 strains tested. Results with the remaining test systems were less consistent and varied with the virus strain. With 3 of the specimens, values obtained in cultures of embryonic chick tissue closely approximated those found in the mice. With the arthropod strain of virus, however, the cultures proved less sensitive than either mice or eggs, an observation which was confirmed in 2 separate tests.

Sharp titration endpoints were usually evident within 2-3 days in cultures of embryonic chick tissue, and in fertile eggs. The endpoints in mice were also apparent in most instances at this time, but occasionally animals, in which the cause of death was later confirmed by subculture, did not succumb until 4 to 12 days after inoculation.

Cultures of monkey renal and human amnion cells were less sensitive than those of embryonic chick tissue. It was also noted that in these cells, addition of large viral inocula frequently was followed by less widespread cellular degeneration. A similar inverse relationship between the size of the inoculum and the extent of cellular degeneration was recently described by Chambers for WEE virus in cultures of strain L cells(11).

From the results of the titrations given in Table II it can be calculated that the volume of viral suspension required to produce infection in the suckling mouse in the case of 3 of the specimens was less by 0.1 to 0.9 log than that necessary to induce cytopathic changes in cultures of chick cells. From the practical standpoint, however, it seemed that this disadvantage of the tissue culture could be overcome by using in this system a larger volume of inoculum. Accordingly, the following experiment was performed:

To each of 20 cultures of chick cells was added in a volume of 0.1 ml 3 TCD₅₀ of virus as calculated from the data obtained in a previous titration of the infected horse brain suspension. The same amount of virus in a volume of 1.0 ml was added to each of 20 similar cultures. In the preparation of the inocula a mixture consisting of 2% inactivated normal horse serum in isotonic buffer solution was employed.

To determine whether the amount of tissue extractions present in a 10% suspension of nervous tissue (as usually employed in isolation attempts) might prove toxic for the cultures or impair their susceptibility to small quantities of virus, a comparable experiment was simultaneously performed in which the virus was diluted in the supernatant fluid from a suspension of normal horse brain prepared in horse serum-isotonic phosphate

TABLE III. Failure of Variation in Volume of Inoculum to Influence Sensitivity of Chick Cell Cultures to Eastern Equine Encephalomyelitis Virus.

| Vol inoculum containing 3 TCD ₅₀ virus (ml) | Chick cell cultures showing cytopathic changes | |
|--|--|---|
| | Virus suspended in horse serum-buffer | Virus suspended in normal horse brain extract |
| .1 | 17/20* | 13/20 |
| 1.0 | 19/20 | 8/20 |

* Cultures with cytopathic changes

Cultures inoculated

buffer solution. At the same time the infectivity endpoint of the virus was also confirmed in 2 separate titrations in which as diluent normal horse brain suspension and horse serum-isotonic phosphate buffer were respectively employed.

The results summarized in the second column of Table III indicate that a 10-fold increase in volume of the inoculum does not diminish the number of cultures that become infected by small doses of virus. A large quantity of brain extractives accompanying the virus may slightly reduce the chances of infection as suggested by the somewhat lower proportion of cultures showing pathogenic changes among those inoculated with virus suspended in normal horse brain extract. It is clear, nevertheless, from these data taken as a whole that a significant increase in the recovery of these agents from specimens of low infectivity may be expected if the volume of the inoculum is increased.

In view of the marked sensitivity of the suckling mouse to strains of this virus, the susceptibilities of cultures prepared from embryonic mouse tissue and newborn mouse brain were investigated to determine whether they might be more effective than those of embryonic chick cells. In Table IV are presented the infectivity endpoints in cultures of murine tissues and in chick cells of human, equine, avian and arthropod specimens. In all cultures a marked and rapidly progressive degenerative effect was observed. The sensitivity of the cultures of embryonic mouse tissues to the virus present in these materials was in general equal to or greater than that of the chick cells.

TABLE IV. Infectivity Titers of Representative EEE Virus Strains in Cultures of Mouse and Chick Cells.

| Cells | Virus source | | | |
|-----------------|--------------|-------------|-----------------|----------------|
| | Human brain | Horse brain | Pheasant brain* | Mosquito pool* |
| Embryonic chick | 6.8† | 4.5 | 8.3 | 4.5 |
| Embryonic mouse | 7.0 | 5.3 | 7.3 | 5.4 |
| Mouse brain | | | N.D. | 5.3 |

* Titrations of 2 samples prepared from the same specimen and tested at different times.

† Negative log ID₅₀/0.1 ml; endpoints calculated by method of Reed and Muench.

Discussion. Despite the recent adoption of tissue culture technics as routine in the primary isolation of many viruses, they have been little used in attempts to recover the virus of eastern equine encephalomyelitis from infected materials of mammalian, avian, or insect origin. Instead, mice and embryonated eggs have been usually employed. In the present investigation, therefore, experiments have been carried out to determine more precisely the value for this purpose of the tissue culture method. The capacity of certain isolated cell systems to detect small amounts of virus in a variety of infected materials was compared with that of embryonated hens' eggs and suckling mice. Although the latter were found to be most efficient in this respect, cultures of trypsinized chick cells and embryonic mouse cells proved nearly as effective.

In practice the size of the inoculum that can be added to the tissue culture may be varied within wide limits. *A priori*, therefore, it would seem possible to render it as effective as the suckling mouse simply by increasing the volume of the inoculum. Indeed by adding in succession several portions of the material under examination one might increase the actual efficiency of the culture far beyond that of the suckling mouse which, of course, can tolerate injection of only small quantities of fluid. If it be accepted that the culture of chick cells is equivalent to the suckling mouse in its capacity to reveal the presence of minimal amounts of virus in specimens of the kind we have studied, then it offers certain advantages over all other systems now available. Thus cultures of chick cells may be prepared quickly and easily and in any number desired from inexpensive and readily available materials. The virus grows rapidly in these cells where it produces cytopathic changes that are

sufficiently characteristic to permit a tentative identification within 24-72 hours. The identity of the agent can then be readily confirmed by subsequent neutralization tests with specific immune serum done in cultures of chick cells. This system can also be conveniently applied to the demonstration of specific antibody formation during convalescence from illness suspected to be caused by the EEE virus as we have found in the study of several patients(12).

The ease with which these cultures may be transported, maintained and inoculated makes them eminently suitable for use in the field for such purposes as epidemiological surveys and studies of host reservoirs, as Sanders has pointed out(13). Finally, their use would eliminate for the investigator the risk of infection inherent in the use of hypodermic needles required in the inoculation of mice.

Summary and conclusions. An evaluation of embryonated hens' eggs, suckling mice and cultures of various tissues for the primary isolation of EEE virus from human, equine, avian and arthropod specimens indicated that suckling mice were the most effective. The evaluation was based on a comparison of the ID₅₀s obtained by concurrently inoculating suckling mice intracerebrally and intraperitoneally, 9-12-day-old embryonated hens' eggs into the yolk sac, and cultures of various tissues with equal quantities of virus. Evidence is presented that cultures of embryonic chick tissue can be made as efficacious as suckling mice for the primary isolation of this virus by increasing the volume of the inoculum. On the basis of these findings and in view of the practical advantages of the tissue culture method it is concluded that cultures of embryonic chick cells should provide a satisfactory technic for use as routine in the etio-

logic diagnosis and in epidemiologic studies of eastern equine encephalomyelitis.

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Effect of Chlorophyll Derivative upon Experimentally Produced Lymphedema in Rats. (23674)

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It has previously been shown(1) that edema of the foot and lower leg in the rat can be produced by injecting a suspension of colloidal kaolin about the ankle. If, at the height of the edema, thorotrast be injected subcutaneously into the dorsum of the foot, dilated lymphatic channels can be demonstrated by roentgenogram. These channels extend upward toward the regional lymph nodes, and it is believed, therefore, that the edema is due to lymphatic block near the lymph nodes. This edema will subside 14-17 days after the initial injection and the foot will appear grossly normal, unless infection involves it, in which case the edema may continue indefinitely, and ulceration may result.

Beef hydrolysates might be expected to exert an effect upon the lymphatic channels comparable with the products of infection. In preliminary studies it was found that no sustained edema appeared following injection of beef hydrolysates into the foot of the rat. However, if the foot had been previously conditioned by an injection of colloidal kaolin, an injection of beef hydrolysate either caused the appearance of edema (if none was originally present) or augmented an already

present edema. Edema due to beef hydrolysate also tended to disappear after 14-17 days, but if repeated injections were given, some chronic swelling might be grossly apparent, or, if not grossly apparent, might be shown by surface area measurements.

Materials and methods. Colloidal kaolin was suspended in 3 times its volume of distilled water. At the beginning of the experiment, 1 cc was injected in the form of a ring completely encircling each ankle. At no time was chlorophyll given along with the kaolin. Beef hydrolysate was prepared from Bacto Beef Powder (Difco). The powder was hydrolyzed in aqueous solution for 3 hours at 37°C, using papain as the digestant. A portion of this was mixed with a derivative of chlorophyll (potassium sodium copper chlorophyllin) so that the resultant solution contained 0.2% chlorophyll by weight. Injections were given into the dorsum of the foot, always paired, so that one foot received the beef hydrolysate without chlorophyll, and the other beef hydrolysate with chlorophyll. When repeated injections were given, the same order was maintained in the feet. The volume of each injection was 0.2 cc.

Healthy male rats were used, 18 in number, 8 being included in series #1, and 10 in series #2. The strain, originally Wistar, has perpetuated itself in our colony for 10 years. All rats weighed at least 200 g. Comparisons were always made between the right and left hind feet of the same animal. The rats in series #1 received one kaolin injection at the beginning of the study, and a second injection 54 days later. Series #2 received but one kaolin injection. Injections of beef hydrolysate (with or without chlorophyll) were given on the 7th, 73rd and 100th day in series #1, and on the 19th and 46th day in series #2. The series was terminated on the 119th day for series #1, and the 63rd day for series #2, at which time surface area measurements were made on the paired legs as follows: Under deep nembutal anesthesia, leg circumference was measured to the nearest mm, beginning at the base of the toes and repeatedly up the leg at 3 mm intervals for a distance of 2.4 cm. These circumferences were then plotted on graph paper against the distances up the leg, and the area thus delimited cut out and weighed on an analytic balance. These weights (of graph paper) were then reconverted to sq mm of leg surface area in the usual way.

Results. Visual observations on the paired legs were made 75 times on the 18 rats within the 14 day period following injections of beef hydrolysate. During this acute phase it was noted that the foot receiving the chlorophyll was less swollen than its mate 31 times, more swollen 3 times, and no difference was noted between the two feet on 41 occasions. From observations made at the time surface areas were measured, it appears that the surface area of one foot-leg must be 9-11% greater than its fellow before a difference can be detected grossly.

Table I shows that there is usually a difference between the chlorophyll treated and non-treated leg, the non-treated leg being the

TABLE I. Effect of Chlorophyll Derivative upon Lymphedema.

| Series # | Surface area of leg segments in mm ² | | Difference in mm ² between 2 legs* |
|----------|---|-------------------|---|
| | No chlorophyll | Given chlorophyll | |
| 1 | 636 | 590 | + 46 |
| | 650 | 590 | + 60 |
| | 646 | 636 | + 10 |
| | 626 | 604 | + 22 |
| | 664 | 628 | + 36 |
| | 640 | 598 | + 42 |
| | 650 | 556 | + 94 |
| | 656 | 620 | + 36 |
| 2 | 610 | 604 | + 6 |
| | 632 | 560 | + 72 |
| | 646 | 570 | + 76 |
| | 648 | 560 | + 88 |
| | 604 | 608 | — 4 |
| | 640 | 624 | + 16 |
| | 552 | 470 | + 82 |
| | 652 | 652 | 0 |
| | 710 | 646 | + 64 |
| | 654 | 590 | + 64 |
| Totals | 11,516 | 10,706 | + 810 |

* + = non-chlorophyll leg is larger; — = chlorophyll leg is larger.

larger. That this is statistically significant* is shown by analysis of paired observations, from the last column of the Table. The computed value of "t" is 6.05, which exceeds the tabular "t" of 3.97 (the value required for significance at the .001 level of probability).

Summary. Under conditions of the experiment (lymphatic block + injection of protein irritants) locally injected chlorophyll diminished the extent of lymphedema, both acutely and chronically. While the mechanism is not established, a heparin-like effect in preventing protein agglutination may be suspected.

* We would express our thanks to Dr. Morris D. Finkner, Biometrician, U. S. Department of Agriculture, Beltsville, Md., for statistical analysis of the data.

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An Hemagglutination Test for Titration of Antibodies to Polioviruses.* (23675)

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It was shown by Bautista, *et al.*(1) that polioviruses were adsorbed to certain human and animal erythrocytes, but that hemagglutination did not occur either in the presence or absence of homologous immune serum. The treatment of red cells with dilute solutions of tannic acid renders these cells capable of adsorbing proteins, and agglutinable in the homologous antiprotein serum(2). In a further extension of this technic, McKenna(3) demonstrated that bovine serum albumin and bovine gamma globulin could be coupled to formalinized erythrocytes, and antibodies to either antigen measured by an hemagglutination pattern.

This report shows that antibodies to polioviruses, which may be considered as protein antigens(4), can be measured by a similar technic.

Materials and methods. Antigen preparations. Seitz-filtered polioviruses, Type I (Mahoney), Type II (MEF-1) and Type III (Saukett) (monkey kidney tissue culture source), were used throughout these studies. The post-filtration infectious titers of these viruses were $10^{6.0}$, $10^{6.8}$ and $10^{6.8}$ TCID₅₀/ml respectively(5). Control monkey kidney cell antigens (MKTCF) were prepared by rapidly freezing and thawing uninfected cell sheets followed by Seitz-filtration. The antigens were concentrated approximately 10-fold by dialysis against 10-20 volumes of a 20% aqueous polyvinylpyrrolidone (PVP) solution at 4°C for 48 hours. Neutralization tests for antibody were performed by use of the metabolic inhibition test against 100 TCID₅₀ of virus(6). *Immune Rabbit Sera.* Twenty albino rabbits each weighing about 3 kg were divided into 4 groups of 5 animals

each. Each group received either 1.0 ml of viable monotypic poliovirus or 1 ml of control antigen intravenously every other day until 5 ml had been injected. Sera obtained one week after the last injection were pooled according to virus type or control and were stored at -40°C. Five other rabbits received 1 ml of trivalent polio vaccine at weekly intervals for three weeks, and sera were obtained 28 days after the first injection. *Immune monkey and human sera.* Both immune monkey and human sera were taken randomly from freezer stocks. Neutralization tests had been carried out previously on all these sera. The monkey sera had been prepared by the intramuscular injection of rhesus monkeys with 1 ml of polio vaccine at weekly intervals for 3 weeks. Immune sera were secured 28 days after the first injection. The immune human sera were taken from individuals who had received one or more inoculations of polio vaccine. *Sheep erythrocytes.* Washed sheep red blood cells were formalinized according to the technic described elsewhere(3). *Experimental.* A 2.5% suspension of formalinized sheep red blood cells was incubated at 37°C for 20 minutes with a freshly prepared 1:20,000 dilution of tannic acid in 0.9% saline. The cells were washed once with saline, centrifuged at 1500 rpm for 5 minutes at 5°C and reconstituted to a 2.5% suspension with saline. Two volumes of these cells then were added to one volume of antigen and one-half volume of 0.15 M phosphate buffered saline at pH 6.4. After incubation at 37°C for 2 hours, the cells were washed once with 1.0% normal rabbit serum in 0.9% saline, and reconstituted to volume with the same diluent. Before freezing the cells at -70°C, infectious titers were determined for the supernates after sensitization of the cells. The initial viral infectious titers, those following PVP concentration, and those of the supernates are

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TABLE I. Infectious Titers before and after 10-Fold PVP Concentration and of Supernates after Sensitization of Red Cells.

| | Type I | Type II | Type III |
|-----------------|--------|---------|----------|
| Initial | 6.0 | 6.8 | 6.5 |
| After PVP | 7.4 | 8.0 | 8.0 |
| " sensitization | 4.5 | 5.8 | 6.0 |

Titers expressed as \log_{10} TCID₅₀/ml.

presented in Table I. It may be seen that over 99% of the virus had disappeared, presumably due to adsorption to the red cells. The viral particles spontaneously eluted from the red cells after standing 2-3 days at 5°C. However, the cells remained agglutinable after storage for 6 weeks in the frozen state. Two-fold dilutions of antisera were made in 0.5 ml amounts in 1.0% normal rabbit serum in saline and 0.15 ml of thawed, sensitized cells was added to each of the serum dilutions. The mixtures were shaken and immediately placed at 1-4°C for 12-18 hours. Hemagglutination titers (HA) were recorded as the highest dilution of antiserum causing agglutination of the cells. All antisera were inactivated at 56°C for 30 minutes, but preliminary work indicated that inactivation was not necessary.

TABLE II. HA Titers* of the Same Immune Rabbit Sera with Cells Sensitized at Different Times.

| Serum prepared to | Serum titers with cells sensitized to | | | |
|-------------------|---------------------------------------|------|------|--------|
| | I | II | III | MKTCT† |
| Type I | 1280 | 160 | 320 | 40 |
| | 1280 | 80 | 320 | 40 |
| | 1280 | 160 | 640 | 20 |
| | 1280 | 40 | 160 | 80 |
| | 1280 | 640 | 640 | 160 |
| GMT‡ | 1280 | 128 | 384 | 56 |
| Type II | 160 | 2560 | 80 | 40 |
| | 160 | 1280 | 160 | 40 |
| | 80 | 1280 | 160 | 10 |
| | 160 | 2560 | 160 | 80 |
| | 160 | 2560 | 80 | 80 |
| GMT | 144 | 2048 | 128 | 40 |
| Type III | 80 | 80 | 1280 | 20 |
| | 80 | 160 | 1280 | 40 |
| | 640 | 80 | 1280 | 40 |
| | 40 | 40 | 320 | 10 |
| GMT | 117 | 80 | 960 | 25 |

Titers expressed as reciprocal of serum dilution.

* HA = Hemagglutinating titers.

† Uninfected monkey kidney tissue culture fluid.

‡ GMT = Geometric mean titer.

Results. The homotypic titer in each instance was appreciably higher than the heterotypic titers when concentrated antigens were used (Table II). These titers represent repeat titrations of the same antisera with cells sensitized at different times. The neutralizing titers of these sera were 1000, 2000 and 640 for Types I, II and III respectively. The 5 rabbits that received the trivalent polio vaccine had the titers shown in Table III, while

TABLE III. HA Titers in 5 Rabbits Given Trivalent Polio Vaccine.

| Rabbit No. | Type I | Type II | Type III | MKTCT |
|------------|--------|---------|----------|-------|
| 256 | 640 | 160 | 320 | 10 |
| 257 | " | 640 | 160 | <10 |
| 258 | 320 | 320 | 320 | 10 |
| 259 | 640 | 160 | 80 | " |
| 260 | 160 | 320 | 160 | " |
| GMT | 420 | 280 | 180 | 10 |

Titers expressed as reciprocal of serum dilution.

the nonspecific agglutination was in all cases 1:10 or less. It is possible that the lower non-specific titers seen in Table III were due to the fewer number of injections. Several weeks later these sera were pooled for neutralization tests. The titers for the pools were as follows: Type I, HA 160, neut. 120; Type II, HA 80, neut. 130; Type III, HA 160, neut. 130.

A comparison of hemagglutinating titers with neutralizing titers in immune human sera is presented in Table IV. It may be seen that sera No. 1, 2 and 3 gave no evidence of HA titers, despite appreciable neutralizing titers. The reason for this lack of agglutination is not evident since all the titrations were made with a single cell lot sensitized to each antigen. A similar comparison in immune monkey sera is shown in Table V.

Discussion. Preliminary experiments using unconcentrated antigens produced low HA titers, but the reaction lacked specificity. By using antigens concentrated about 10-fold with PVP, the homotypic titers were approximately 10 times higher than the heterotypic titers, and about 20-50-fold higher than the uninfected tissue control. Since no soluble component has been demonstrated in or from polioviruses, one can only surmise that the

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TABLE IV. Comparison of HA and Neutralizing Titers of Immune Human Sera.

| Serum No. | Type I | | Type II | | Type III | | MKTCF HA |
|-----------|--------|--------|---------|-------|----------|-------|-------------|
| | HA | Neut.* | HA | Neut. | HA | Neut. | |
| 1 | <10 | 80 | <10 | 80 | <10 | 68 | <10 |
| 2 | " | 40 | " | 1280 | " | 136 | " |
| 3 | " | 416 | " | 1088 | " | 1664 | " |
| 4 | 10 | <10 | 40 | 68 | 640 | 2560 | " |
| 5 | 40 | 160 | 10 | 13 | 40 | 272 | " |
| 6 | 80 | 104 | 640 | 640 | <10 | 20 | " |
| 7 | 160 | 40 | 1280 | 2560 | 1280 | 2560 | " |
| 8 | " | 640 | " | 1280 | 160 | 80 | 10 |
| 9 | 320 | 416 | " | 2176 | 1280 | 2560 | <10 |
| 10 | 640 | 2560 | 160 | 208 | 2560 | " | 10 |
| 11 | " | 2176 | 1280 | 832 | 10 | <10 | <10 |
| 12 | " | 832 | 640 | 1280 | 160 | 640 | " |
| 13 | " | " | 1280 | 2560 | 1280 | 2560 | 10 |
| 14 | " | 1664 | " | 1280 | 320 | 1280 | " |
| 15 | " | 1280 | 640 | 1664 | 640 | 2176 | <10 |

* Neutralizing titers expressed as geometric means of triplicate titrations.
Titers expressed as reciprocal of serum dilution.

entire viral particle became attached to the red cells.

Approximately a 2-5% antigenic cross has been observed with some lots of purified virus when measured by a 50% CF test(7); the average cross as measured by HA was about 12%. This increased percentage may be due to the increased sensitivity of the HA over the CF test, since it has been shown that the HA test is about 35 times as sensitive as the 50% CF test using a bovine gamma globulin-anti-BGG rabbit serum system (unpublished data).

TABLE V. Comparison of HA and Neutralizing Titers of Immune Monkey Sera.

| Serum No. | Type I | | Type II | | Type III | |
|-----------|--------|--------|---------|-------|----------|-------|
| | HA | Neut.* | HA | Neut. | HA | Neut. |
| Normal | 2 | <2 | 2 | <2 | <2 | <2 |
| 1 | <2 | 4 | <2 | 23 | 4 | 2 |
| 2 | 2 | 10 | 8 | 13 | <2 | 2 |
| 3 | 4 | 3 | 8 | 4 | 8 | 4 |
| 4 | 8 | 3 | 64 | 208 | 16 | 10 |
| 5 | 8 | 10 | 32 | 23 | <2 | 8 |
| 6 | 10 | 9 | 20 | 16 | <10 | 2 |
| 7 | 16 | 10 | <2 | 2 | <2 | 8 |
| 8 | 20 | 36 | 80 | 12 | 40 | 26 |
| 9 | 20 | 49 | 80 | 128 | <20 | 10 |
| 10 | 80 | 8 | 320 | 320 | <2 | 8 |
| 11 | " | 12 | " | 332 | <10 | 9 |
| 12 | " | 32 | 160 | 16 | " | 2 |
| 13 | " | 49 | 320 | 64 | 160 | 97 |
| 14 | " | 64 | " | 102 | " | 128 |
| 15 | " | 256 | 80 | 416 | 40 | 51 |
| 16 | " | 1331 | 320 | 24 | 160 | 256 |
| 17 | 160 | 128 | " | 49 | 40 | 140 |

* Neutralizing titers expressed as geometric means of triplicate titrations.

Titers expressed as reciprocal of serum dilution.

The basic technic as described by Boyden (2), with the formalin modification developed in this laboratory, has proven quite useful to us. However, many factors can influence the outcome of any given titration. The scheme depends on protein adsorption, and the sensitized cells may at times spontaneously agglutinate in high protein concentrations. In this particular instance, 4 of the 15 human sera tested agglutinated cells sensitized to MKTCF to a titer of 1:10. Since all of these sera were from people who had received polio vaccine, the agglutinating titer to MKTCF may have been due to anti-monkey antibodies in these sera. This HA to monkey kidney material is seen also in Tables II and III. However, normal monkey sera showed no agglutination with cells sensitized to any of the 3 viral types. All non-dialyzable material, including kidney proteins, are concentrated by the PVP procedure. It would not be expected that monkeys would form antibodies regularly to homologous kidney proteins with the immunizing schedule used here. It is therefore felt that, when using sera other than monkey, the test is not reliable for the detection of antibodies to polioviruses in serum dilutions <1:10. Chicken serum was unsuitable for assay purposes since spontaneous agglutination of the sensitized cells occurred.

When cells were sensitized to purified Type III virus(7) and monovalent immune rabbit sera were titrated with these cells, homotypic titers averaged 1:320 while the heterotypic

TABLE VI. Molecules of Antigen Used for Sensitization.

| Antigen | γ /ml | Molecular wt | Molecules per ml |
|--------------------------|-----------------|-------------------|----------------------|
| Ovalbumin | 5×10^8 | 4×10^4 | 7.5×10^{10} |
| Human γ -globulin | 100 | 1.6×10^5 | 3.8×10^{14} |
| Polio I | 13 | 6.7×10^6 | 1.2×10^{12} |
| II | 5 | <i>Idem</i> | 4.5×10^{11} |
| III | 5 | " | <i>Idem</i> |

Type I, 64 CF units/ml; Type II, 25 CF units/ml; Type III, 25 CF units/ml.

titers were 1:40. Nonspecific titers were <1:10. These results suggest that cross reactions were not lessened when purified virus was used to sensitize the cells, but nonspecific titers were lessened.

Data showing the number of molecules used to sensitize the cells are given in Table VI. These calculations were based on the relationship of 5 CF unit/ γ of viral antigen (J. Charney, personal communication) while the amounts for the ovalbumin and gamma globulin were taken from Boyden(2). It becomes evident that the higher the molecular weight of the antigen, the fewer number of molecules are required for sensitization.

Statistically, the overall correlation between HA and neutralizing titers was fairly good ($r = 0.75$). However, a statistical analysis is an inadequate criterion when sera do not show any HA titers despite appreciable

neutralizing antibody. It should be emphasized that if a serum has no HA titer, this does not mean that the serum contains no neutralizing antibodies (Table V, sera 1, 2 and 3).

Summary. An hemagglutination technic for the titration of antibodies to polioviruses has been described. The HA titers showed approximately a 75% correlation with neutralizing antibody. The virus-sensitized cells did not remain agglutinable for more than 2 days at 5°C, but have proven stable for at least 6 weeks when kept frozen. An antigenic cross among viral types of 12% was observed. Purified virus lessened the nonspecific HA, but not the percentage of cross reactions.

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Relationship of Mental and Emotional Stress to Serum Cholesterol Levels.* (23676)

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(Introduced by U. D. Register)

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With the increased incidence of coronary heart disease there has been much speculation concerning a possible cause-and-effect relationship between stress, cholesterol-lipid-lipoprotein metabolism and coronary atherosclerosis(1,2,8). In considering the pathogenesis of a disease such as atherosclerosis or myocardial infarction one must recognize the pos-

sibility of direct as well as indirect effects of such factors as stress, diet, sex, age, exercise, etc. This is particularly evident in the case of stress because of its varied nature and the wide spectrum of bodily responses attributed to it(3). That certain forms of physiological stress can elevate serum cholesterol has been demonstrated. In a study of 55 male subjects Kuhl, *et al.*(4) found that brief immer-

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TABLE I. Distribution of Subjects According to Serum Cholesterol Changes under Stressful Conditions.

| Groups* | Diff. between total cholesterol levels for stress and control periods, mg % | Avg total cholesterol, mg %† | | No. of subjects | % of total No. subjects | Significance of diff. from total group (P values) |
|---------|---|------------------------------|---------------|-----------------|-------------------------|---|
| | | Control period | Stress period | | | |
| I | <9 | 218 ± 7.1 | 216 ± 9.0 | 14 | 32 | >.1 |
| II | + 10-15 | 210 ± 7.3 | 223 ± 7.3 | 10 | 23 | <.1 |
| III | + 16-137 | 213 ± 7.3 | 261 ± 12.2 | 20 | 45 | <.01 |
| Total | + 24 | 214 ± 3.5 | 238 ± 4.8 | 44 | 100 | <.001 |

* The groups are arbitrarily divided with respect to the stand. error of the difference of the means which equals 5.90 mg %.

† Values shown are mean ± stand. error of mean.

sion in cold water was followed by an immediate and significant increase in serum cholesterol. Selye(5) has described a biphasic response of serum cholesterol and lipids in his adaptation syndrome. However, in an attempt to evaluate the influence of "mental stress" Beischer(6) failed to find any significant changes in concentrations of cholesterol, phospholipids, or lipoproteins in the serum following the subjects' first experience of a human centrifuge ride or the experience of simulated failure of a low pressure chamber. Further investigation of this aspect of the problem has therefore seemed advisable.

In the present study 44 male students were studied during periods in which they were free of examinations as well as during an examination week. Thus the purpose of this study was to determine what possible effect mental and emotional stress might have on the level of serum cholesterol.

Method. All those participating in this study were apparently healthy, male, medical students whose ages ranged from 21 to 37, the mean age being 25. The subjects continued their usual program with no major changes in their diet or exercise for the period of observation. All blood samples were collected in the post-absorptive state. Three control days were chosen to come within a period of time in which the students were free of examinations for at least a week before and after the samples were taken. Samples were drawn on the mornings of 4 examination days to determine the effect this form of mental and emotional stress might have on serum cholesterol. Total serum cholesterol was determined in duplicate on each sample by the method of

Pearson, Stern, and McGavack(7).

Results. The mean value for serum cholesterol in 44 subjects on the 3 control days was 214 mg %. The mean level of serum cholesterol during the examination days was 238 mg %, which represents an average increase of 11% over the control period. This increase in mean value of serum cholesterol under these conditions of mental and emotional stress is statistically significant ($P < 0.001$).

The 44 subjects were arbitrarily divided into 3 groups with respect to the standard error of the difference of the mean levels of serum cholesterol during the control days and the examination days. Those subjects showing a change less than 9 mg % from their control levels were placed in Group I. Group II includes those subjects in whom serum cholesterol increased 10-15 mg % above the subject's control level. Group III includes those individuals showing an increase of 16 mg % or more above their control values. By reference to the statistical findings for the entire group of 44, Group I can be considered to have a probability ($P > 0.1$) of being due to chance; Group II ($0.1 > P > 0.01$); Group III ($P < 0.01$). Table I shows the distribution of subjects according to their serum cholesterol changes.

There were some individuals in Group III who experienced a remarkable increase in their serum cholesterol. One particularly notable example averaged 259 mg % for the control samples, but this average increased to 395 mg % during the 4 examination days. On 2 of these days his serum cholesterol reached levels of 520 mg % and 536 mg %.

Since life itself is stressful and since this

experiment was conducted during the regular school term it is obvious that these control data cannot represent truly an unstressed level of serum cholesterol. It does seem likely, however, that the periods of observation do coincide with periods of relatively lesser and greater degrees of mental and emotional stress.

As one might expect, these individuals vary greatly in their clinical and biochemical responses to stress. It is apparent that the situational stress of examination week must have different meaning and hence a differing stress value for the subjects. Further investigation is needed to establish whether this increase in serum cholesterol is harmful or simply part of a normal and desirable bodily adjustment to mental and emotional stress.

The difference between these results and those of Beischer(6) may be due, as he has suggested, to his use of fewer subjects and of a particularly and relatively transient stress experience. Our data would coincide with changes which Selye(5) has described in connection with the "resistance phase" of the general adaptive response to various forms of

systemic stress.

Summary. A significant increase in mean value for serum cholesterol is shown to accompany the mental and emotional stress of examination week in a group of 44 apparently healthy, male, medical students. The mean value for serum cholesterol increased to 238 mg %, which represents an 11% increase over the mean control value of 214 mg % ($P < 0.001$).

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Hormonal Regulation of Pituitary Adrenocorticotrophin.* (23677)

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Most of the information available concerning regulation of pituitary secretion of adrenocorticotrophin (ACTH) has been derived from indirect indices of pituitary function, primarily depletion of adrenal ascorbic acid in the intact rat. On the other hand, direct determinations of the ACTH content of pituitary tissue under various experimental conditions have been reported infrequently, and, when attempted, have often been conflicting or inconclusive(1-11).

Recently, Saffran and Schally(12) have de-

scribed a technic of biological assay for ACTH based upon steroidogenesis by adrenal slices *in vitro*. Birmingham *et al.*(13) have reported a new method of extraction of pituitary ACTH. Assay of their extracts by the *in vitro* technic yielded values considerably higher than those obtained previously. These latter procedures have been employed in the following study, which was undertaken to define more clearly the effects of adrenalectomy and injection of ACTH, epinephrine, and cortisone upon pituitary ACTH content.

Materials and methods. All animals were kept in a constant temperature room for at least one week prior to use and maintained on a diet consisting of Purina Laboratory Chow and water *ad libitum*. Adrenalecto-

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TABLE I. Frequency Distribution of Index of Precision (Λ *) for 100 Bioassays of ACTH.

| Λ | .01-.05 | .05-.10 | .10-.15 | .15-.20 | .20-.25 | .25-.30 | .30-.35 | .35-.40 | .40-.45 |
|-----------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Frequency | 1 | 21 | 30 | 26 | 6 | 9 | 5 | 1 | 1 |

* Λ = assay error/slope.

mized rats received a supplement of 1% saline and 5% glucose in the drinking water. Male rats, 120-140 g, of the Sherman strain, were randomly divided into 6 groups and treated as follows: 1) 0.9% saline, 0.2 ml, injected s.c. once daily; 2) ACTH gel, 4 U.S.P. units in 0.1 ml, injected s.c. once daily; 3) cortisone acetate, 5 mg in 0.2 ml, injected s.c. once daily; 4) epinephrine in oil, 1:500, 0.1 ml, injected s.c. twice daily; 5) untreated; and 6) adrenalectomy without further treatment thereafter. The animals were injected for 7 days and sacrificed under Nembutal anesthesia 24 hours after the last injection. Body weights and weights of the adenohypophysis and the adrenal glands were measured. The individual pituitary glands were promptly extracted by the "modified glacial acetic acid method" of Birmingham *et al.* (13). Each extract was assayed for ACTH by a modification of the *in vitro* technic described by Saffran and Schally (12). The adrenal glands from each of 8 female rats, 120-140 g, were removed, cleaned and cut into eighths[§] with razor blades. The 16 pieces from each rat were distributed randomly among 16 squares drawn on moistened filter paper. The 8 pieces in each square were weighed together, transferred to 25 ml Erlenmeyer flasks each containing 2 ml of Krebs-Ringers-Bicarbonate solution (KRB) made up with 1.83% rather than 1.22% CaCl_2 (14). The tissue was incubated with shaking for 30 minutes at 37°C under an atmosphere of 95% O_2 and 5% CO_2 . The medium was removed by suction and the pieces washed with 1 ml of KRB. The wash solution was discarded and 2 ml of fresh KRB added to each flask. The assay design then permitted a standard ACTH and each of 3 pituitary extracts to be added to the flasks at 2 dose levels in duplicate. The

flasks were incubated for 2 hours under the conditions already described. The medium was transferred to 15 ml glass-stoppered centrifuge tubes each containing 2.5 ml of methylene chloride. The adrenal pieces were washed with 0.5 ml of KRB and the washings added to the original medium. The tubes were shaken 200 times and centrifuged at 3500 rpm for 2 minutes. A 2 ml aliquot of the methylene chloride layer was evaporated to dryness under air in round-bottomed tubes, 1 ml of absolute redistilled methanol was added and UV absorption at 225, 240, and 255 $\text{m}\mu$ measured in a Beckman DU spectrophotometer against a blank extracted from KRB alone. The Allen correction (15) was applied to the peak reading at 240 $\text{m}\mu$ and the data analyzed according to the methods of Bliss (16).

Results. One hundred bioassays of ACTH employing the *in vitro* technic have been performed. The index of precision (Λ) of each of these assays, defined as the ratio of each assay error to its corresponding slope, is presented in Table I. The median falls within the range between 0.10 and 0.15 and is comparable to values reported by authors who have employed a similar technic (12,14,17) and to those obtained by other methods. Maintenance of the animal quarters at a constant temperature was found to be the most important variable affecting assay precision. Even minor and transient variations in temperature were associated with a significant loss of precision; the values of Λ greater than 0.25 were obtained before this observation was fully appreciated.

The log dose-response curve under the conditions of the assay was demonstrated to be a straight line between doses of 8 milliunits (μu) and 256 μu of ACTH per 100 mg adrenal weight. Both the doses of standard ACTH and the assumed doses of each pituitary extract were selected within this range. Eight assays were rejected because their combined

[§] Recently, the capacity of the assay has been enlarged by cutting the adrenals in twelfths with distribution in 24 flasks, thus permitting simultaneous assay of a standard and 5 unknown preparations, without loss of precision.

TABLE II. Comparison of *In Vitro* Bioassay to Ascorbic Acid Depletion Technic and Results of Repeated Assays.

| Preparation | Label potency | Measured potency No. 1 | 95% confidence limits | Measured potency No. 2 | 95% confidence limits |
|-----------------------|---------------|------------------------|-----------------------|------------------------|-----------------------|
| Organon (No. 9560) | 42.8 u/mg* | 36.6 u/mg | 19 - 54 | | |
| " (No. 511R) | 550 mu/mg† | 546 mu/mg | 235 - 754 | | |
| Armour (commercial) | 1 u/mg* | 1.1 u/mg | .6- 2.7 | 1.0 u/mg | 0.5- 2.3 |
| Rat pituitary extract | | 75 mu/mg | 38 -142 | 72 mu/mg | 36 -135 |

* Assayed by ascorbic acid depletion technic.

† Assayed by the Saffran and Schally technic.

slopes were not significant at the 1% level of confidence. No assay required rejection owing to significant non-parallelism among preparations, although, occasionally, a single unknown was so rejected and re-assayed on the following day.

The validity and reliability of the assay technic were determined by comparison of the potencies of several ACTH preparations to those obtained by the ascorbic acid-depletion method and by re-assay of the same unknown. The results summarized in Table II, indicate both satisfactory validity and reliability with a maximum error of 15%.

The results of the bioassays of pituitary glands of the 56 animals subjected to the various experimental conditions included in the present study are summarized in Table III. Both the mean ACTH concentration (mu/mg of wet pituitary tissue) and the mean ACTH content (mu per gland) are presented for each treatment group. The ACTH values represent semi-weighted means and their approximate standard errors ($\text{Antilog } \bar{M} \pm 2.303 s_{\bar{M}}$ $\text{Antilog } \bar{M}$) (16). The actual statistical comparisons by *t* test were based upon the mean log potencies and their exact log

errors ($\bar{M} \pm s_{\bar{M}}$) for each group. Rigorous comparisons were ensured by derivation of the number of degrees of freedom from the number of *M*'s in each group rather than the number available in each *s_M*. Each treatment group was compared with the saline-injected control group, with the exception of the adrenalectomized group which was compared with the untreated control group, since the adrenalectomized animals received no injections.

Significant and comparable depletions in pituitary concentration and content of ACTH were obtained after injection of either cortisone or epinephrine for a period of 7 days. The fall in ACTH in both these groups exceeds 60 percent when compared to the saline-injected control group. As anticipated, cortisone administration was associated with a statistically significant decrease in adrenal weight, whereas epinephrine injection was followed by significant adrenal hypertrophy.

ACTH administration, on the other hand, produced a significant increase in both pituitary concentration and content of ACTH. The concentration was almost double that obtained for the saline-treated group and content was 1.67 times greater. Adrenal hypertrophy was

TABLE III. Effects of Various Treatments on Pituitary ACTH Concentration and Content, Pituitary Weight and Combined Adrenal Weight.

| Treatment group | No. of animals | ACTH conc. (mu/mg)* | ACTH content (mu)* | Pituitary wt (mg)† | Adrenal wt (mg)†‡ |
|---------------------|----------------|---------------------|--------------------|--------------------|-------------------|
| Saline (control) | 9 | 41 ± 6 | 224 ± 35 | 5.6 ± .3 | 31 ± 1 (10) |
| ACTH | 9 | 80 ± 6§ | 364 ± 26 | 5.1 ± .4 | 41 ± 1§ (5) |
| Cortisone | 9 | 17 ± 3§ | 66 ± 20§ | 4.8 ± .3 | 20 ± 2§ (6) |
| Epinephrine | 9 | 16 ± 2§ | 91 ± 13§ | 5.5 ± .1 | 36 ± 1§ (8) |
| Untreated (control) | 13 | 63 ± 12 | 319 ± 49 | 5.1 ± .4 | |
| Adrenalectomy | 7 | 102 ± 9 | 749 ± 60§ | 7.0 ± .2§ | |

* $\text{Antilog } \bar{M} \pm 2.303 s_{\bar{M}}$ ($\text{Antilog } \bar{M}$).

animals studied indicated in parentheses.

|| Significant at the 3% level of confidence.

† Mean ± stand. error of mean.

‡ No. of

§ Significant at the 1% level of confidence.

|| Significant at the 5% level of confidence.

observed also, consistent with the dose of ACTH employed. The pituitary weights of the group treated with ACTH, cortisone or epinephrine did not differ significantly from those of the control group injected with saline.

Adrenalectomy resulted in a barely significant increase in pituitary ACTH concentration; however, a highly significant increase in total ACTH content was observed, well over double that of the untreated control group. The marked pituitary hypertrophy observed after adrenalectomy contributed to this increase in ACTH content. Although the mean pituitary ACTH content of animals treated with saline appeared to be 30% lower than the mean content in untreated animals, this difference was not statistically significant. Finally, the significance of all the comparisons described was unaltered when variations in body weight were taken into account.

Discussion. Measurements of pituitary ACTH concentration and content, such as those performed in the present study, permit varying interpretations. For example, increased content and concentration of ACTH may mean increased ACTH synthesis or increased storage or both. Conversely, decreased concentration or content may indicate decreased ACTH formation or decreased storage, *i.e.*, increased ACTH secretion. The following discussion must be viewed with these possibilities in mind.

The ACTH content of the pituitary glands of untreated rats as determined in the present study is significantly less than that reported by Birmingham *et al.* (13) who employed an identical procedure for extraction and a similar bioassay technic. This difference cannot be explained on the basis of the available evidence, but may be due, in part, to differences in the strain of rat studied (unspecified by the previous authors). However, the values reported significantly exceed those obtained by other methods of extraction and assay (3,11, 18).

Saline injection apparently resulted in a 30% fall in pituitary ACTH content when compared with uninjected control animals, but this difference was not statistically significant. However, the data suggest that repeated injections *per se* may result in moderate pitui-

tary ACTH depletion; the study of more animals may be required to establish this difference.

The observation that adrenalectomy is followed by a significant rise in pituitary ACTH content associated with pituitary hypertrophy confirms previous reports (3,11) and is consistent with the hypothesis that a decrease in the circulating level of adrenal steroids is followed by accelerated production of ACTH. Conversely, an increased steroid level, produced by cortisone administration, was followed by depletion of pituitary ACTH. The associated adrenal atrophy suggests that ACTH secretion was also decreased. These findings suggest that cortisone administration for 7 days reduced ACTH synthesis with concomitant diminution of ACTH secretion. They are in accord with the report of Farrell and Laqueur (1) that cortisone injection is followed by pituitary ACTH depletion in dogs.

Epinephrine administration for 7 days is associated with significant depletion of pituitary ACTH similar to that obtained with cortisone. However, adrenal hypertrophy, presumably due to increased ACTH secretion, also was observed in contrast to the cortisone-induced adrenal atrophy. No statement concerning the effect of epinephrine upon the rate of ACTH synthesis can be made with the data currently available.

The significant rise in pituitary ACTH content following ACTH administration is surprising. The expected finding would have been pituitary ACTH depletion similar to that found in the cortisone-treated animals, since the only commonly accepted effect of ACTH is stimulation of the adrenal cortex. The increase observed is of such magnitude that it cannot be explained by the possibility of contamination of the extracted pituitary glands with blood containing even large amounts of ACTH. Experiments in progress involving administration of ACTH to adrenalectomized rats indicate that this increase in pituitary ACTH is not mediated by the adrenal gland. At least 3 alternative explanations are suggested: 1) increased blood ACTH may inhibit pituitary ACTH release; 2) an increased circulating level of ACTH may stimulate ACTH synthesis; or 3) the pituitary

gland adsorbs ACTH when present in the blood in increased concentration. Gemzell(5) has claimed that administration of ACTH for one week to adrenalectomized rats was followed by no change in pituitary ACTH content. His results are not comparable to those herein described since his technics(3) of pituitary ACTH extraction and assay yielded values 80% lower than those obtained in the present report.

Summary. A modification of the Saffran and Schally technic of *in vitro* bioassay of ACTH is described and evidence is presented for its precision, validity and reliability. Changes in pituitary ACTH content and concentration in rats after administration of various hormones for one week were studied by means of this technic. ACTH administration and adrenalectomy both resulted in a striking increase in pituitary ACTH content. Epinephrine and cortisone administration were both followed by significant depletion of pituitary ACTH.

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Demonstration of Differences Between Normal and Tumor-Bearing Animals.* (23678)

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If it is assumed that tumors are metabolically or otherwise different from the normal host tissue, this distinction should express itself qualitatively or quantitatively in some of the intermediate or terminal biochemical products of malignant growth. A simple experimental design has been employed to demonstrate such differences in animals bearing transplanted tumors of various types in comparison with otherwise identical normal con-

trol animals. The principle of the procedure involves the challenging of tumor-bearing and normal mice with an intraperitoneal lethal dose of an appropriate compound capable of reacting *in vivo* with one or more of the unique metabolites associated with the tumor in question. The effect of this reaction on the survival times of the 2 groups of animals is then determined. Dissimilarity in survival times is presumably due to a decrease or in-

crease in the toxicity of the administered compound which, in turn, is thought to be an expression of its selective binding or chemical alteration by the tumor or its products. This measurable difference, as well as other variations in response, reflects the biochemical differences in the tumor-bearing hosts as contrasted with the controls.

The mouse melanoma has been employed as the primary model since some of its aberrant metabolic products and reactions are known. For example, 3,4-dihydroxyphenylalanine (DOPA) combines *in vitro* with *p*-phenylenediamine (PPDA) in an oxygen-consuming, pigment-producing reaction(1-4). DOPA is an accepted component produced in the biochemical chain of events in the metabolism of pigmented melanomas(5), and thus a rational foundation is provided for an *in vivo* reaction when PPDA is administered to melanoma-bearing animals. The consequence of this procedure, with the above combination, is a significant difference in the mean survival times of the two groups with a protective effect provided by the presence of the tumor. This phenomenon can be inverted by changing the administered compound or by employing a different histological or physiological type of tumor, in which event the tumor may become a biochemical liability to the host.

Materials and methods. The Cloudman S91 mouse melanoma(6), the Ehrlich (solid) carcinoma(7), and Sarcoma 180(8) were

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some of the tumors tested and are used illustratively for this brief report. The compounds reported include 3,4-dihydroxyphenyl-*DL*-alanine (DOPA), from Nutritional Biochemicals Corporation; *p*-phenylenediamine (PPDA), from Amend Drug Co. and Matheson, Coleman & Bell; N, N-dimethyl-*p*-phenylenediamine (DPP), from Eastman Organic Chemicals; and O-diazoacetyl-*L*-serine (Azaserine) and its analog, 6-diazo-5-oxo-*L*-norleucine (DON), from Parke, Davis & Co. The doses of the compounds are given in the text for the specific experiment but in general they were approximately 2 or 3 times the LD₅₀. All compounds were given as a single intraperitoneal dose on a milligram per kilo basis, including the weight of the tumor in the experimental group. Each mouse was weighed individually to 0.1 g for dose determination. The ratio in ml of the volume injected was 1/100 the weight of the animal. For convenience of dose calculation and injection the following standard procedure was employed. One hundred mg of compound was dissolved or suspended in 10 ml of water to give a dose of 100 mg/kg in a 20-g mouse when injected with 0.2 ml of solution. A 25-g mouse received 0.25 ml, etc. The dose volume was kept approximately constant for all compounds and the different requirements of milligrams per kilo to yield a lethal dose were controlled by an appropriate increase or decrease of drug concentration. All experiments were carefully balanced with regard to such tangible variables as time factors, and sex, strain, weight, and age of animals. To rule out or to detect the influence of time or of oxidation on the test solution, the experimental and control animals were injected alternately. When 2 or more tumor types or 2 or more compounds were being compared, appropriate alternation sequences were employed. The animals were identified by sequential numbering at the time of injection. Injection and death times were recorded to the nearest minute. Following death, the animals were reweighed and the tumors carefully removed to provide information on comparative tumor and carcass weights. For simplified analysis of the results the survival times were calculated to the nearest minute, the

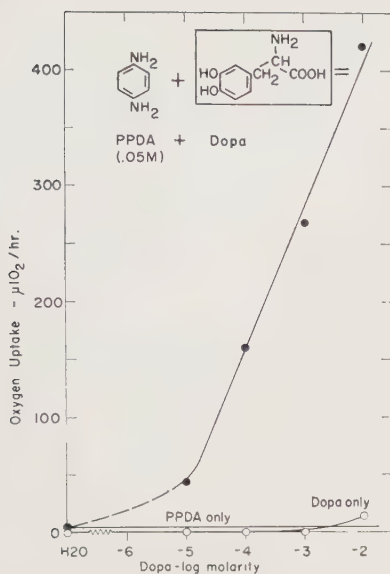


FIG. 1. Synergistic oxidation between 3,4-dihydroxyphenylalanine (DOPA) and *p*-phenylenediamine (PPDA) *in vitro*. PPDA concentration was constant at 0.05 *M* while DOPA was increased logarithmically.

data of the normal controls and the tumor-bearing mice were segregated, and the individual values within each group were arrayed in order of survival rank. For purposes of plotting the data and visualizing the family response of each group, cumulative averages have been employed. This procedure reduces the influence of one or 2 aberrant animals whose survival response is atypical. Thus, the first point for each group on the graph represents the shortest survival time of a single animal, while the next point represents the average survival time of the first 2 mice to die, etc. The last point represents the average survival time of all mice in its respective group. The midpoint on the curve, or the median average of the group response, is a more stable single value than the total average since, again, when the survival times are ranked, the median is independent of a small number of atypical longtime survivors. The experiments illustrated have in most cases been repeated several times with similar findings. Where statistical significance is of consequence it is discussed with the experimental results.

Results. Fig. 1 illustrates the manometric

oxygen-consuming reaction that takes place when DOPA (3,4-dihydroxyphenyl-DL-alanine) and PPDA (*p*-phenylenediamine) are combined *in vitro*. It is seen that, under the conditions of the experiment, neither component oxidizes appreciably by itself but upon combination there is a vigorous oxygen uptake comparable to that produced by a potent enzyme preparation. In the illustrated experiment PPDA was held constant at 0.05 *M* while DOPA concentration was increased logarithmically to yield a straight line function through its solubility range. It is of consequence to note that a substantial uptake is still obtained when DOPA is present at the relatively low concentration of 1×10^{-4} *M*. A brown melanin-like pigment is the main product of the reaction and is less toxic to mice than the original components.

The protective effect of the S91 Cloudman mouse melanoma against the toxicity of PPDA is demonstrated in Fig. 2. The difference in the response between the two groups is highly significant and could occur by chance in less than 1 out of 100 cases ($P = 0.006$). The experiment was carried out with a uniform group of DBA/2 back-cross female mice approximately 3 months old, with the only tangible difference between the 2 groups being the presence of melanoma which had been implanted 40 days previously. Both groups appeared to be in good physical condition. The normal controls had an average weight of 20.6 g and the tumor-bearing mice an average of 21.8 g. The tumors ranged in weight from 4.0 to 7.6 g with an average of 5.5 g. At death, when the tumors were removed, the carcass weights averaged 16.3 g.

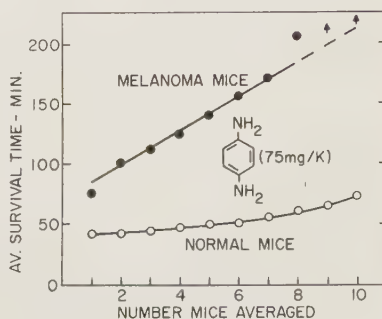


FIG. 2. Protective effect of Cloudman S91 melanoma against toxicity of *p*-phenylenediamine (PPDA).

TABLE I. Comparative Survival Time Response of Normal and Melanoma-Bearing Mice when Challenged with a Lethal Dose of PPDA (75 mg/kg).

| | Normal control mice | Tumor-bearing mice | T/N ratio* |
|---------------|---------------------|--------------------|------------|
| No. of mice | 10 | 10 | |
| Survival time | | | |
| Avg | 72 | 289 | 4.0 |
| Range | 42-144 | 76->640 | |
| Stand. dev.† | 30 | 195 | |
| Median | 60 | 222 | 3.7 |
| Avg median‡ | 50 | 148 | 3.0 |
| Avg mouse wt | 20.6 | 21.8 | |
| " carcass " | 20.6 | 16.3 | |
| " tumor " | | 5.5 | |

* Survival time ratio between the normal control and tumor-bearing mice.

† P = 0.006.

‡ Median point on the curves employing cumulative averages such as Fig. 2. This is the mean response of the first 50% of the animals in each group.

The experiment illustrated in Fig. 3 shows the dependence of average survival time upon tumor size. This experiment is similar to the preceding one but compared groups of small, medium, and large tumors with normal controls. The average tumor weights were 0.74, 1.81, and 5.6 g, with 10 mice in each tumor category and a total of 30 control animals. The differences are highly significant statistically and demonstrate the existence of a quantitative relationship between PPDA toxicity and the protective effect of melanoma in mice.

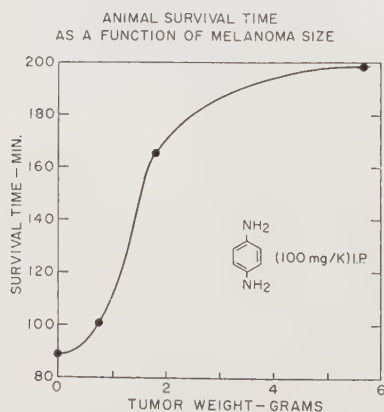


FIG. 3. Increasing protective influence of S91 melanoma as a function of tumor size against relative toxicity of an LD₁₀₀ dose of *p*-phenylenediamine (PPDA).

SIMILAR SURVIVAL FOLLOWING INJECTION-DPP

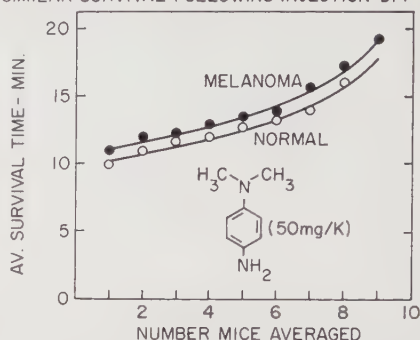


FIG. 4. Non-protective effect of S91 tumor against toxicity of *N,N*-dimethyl-*p*-phenylenediamine (DPP).

If the hydrogens of one of the amino groups of PPDA are replaced by methyl groups to give *N,N*-dimethyl-*p*-phenylenediamine (DPP), the protective effect of the pigmented tumor against the compound's toxicity is lost (Fig. 4). Similar results were obtained with the nonpigmented S91 amelanoma. In a preliminary experiment with the same compound, animals bearing the Ehrlich carcinoma succumbed much sooner than did their normal counterparts to a lethal dose of DPP. When the Ehrlich tumor was tested with

SIMILAR SURVIVAL FOLLOWING A SINGLE DOSE OF PPDA

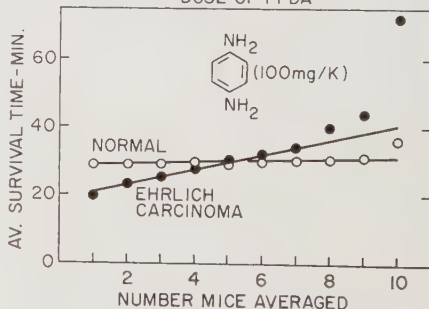


FIG. 5. Non-protective effect of Ehrlich carcinoma against toxicity of *p*-phenylenediamine (PPDA).

PPDA, however, in contrast to the differential toxicity obtained with melanoma, neither tumor-protective nor liability effects were observed (Fig. 5). It is thus seen that the various effects are dependent upon tumor type as well as upon compound structure.

Diaminodiphenylamine (DADPA) reacts with DOPA in the Warburg apparatus similarly to PPDA in that oxygen is consumed and pigment is formed. However, when

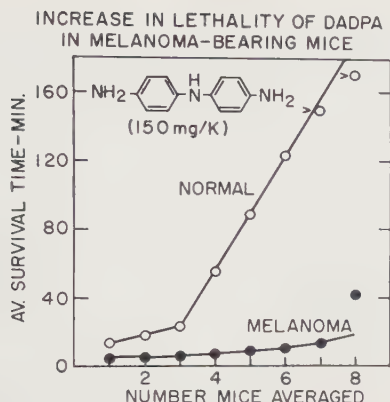


FIG. 6. Increased toxicity of diaminodiphenylamine (DADPA) in S91 tumor-bearing mice compared with normal controls.

tested against melanoma *in vivo* (Fig. 6), the opposite toxicity effect is obtained. Thus, with this compound, the tumor represents a liability rather than a protection to the organism, which indicates that the product resulting from DADPA and melanoma components is more—rather than less—toxic to the organism than the analogous products resulting from PPDA.

A striking difference in susceptibility to the acute toxic effects of Azaserine between normal Swiss mice and those carrying Sarcoma 180 is shown in Fig. 7. The average median survival time of the normal control animals was 598 minutes compared with 205 minutes for the tumor-bearing mice. Here again the presence of the tumor increases the toxic ef-

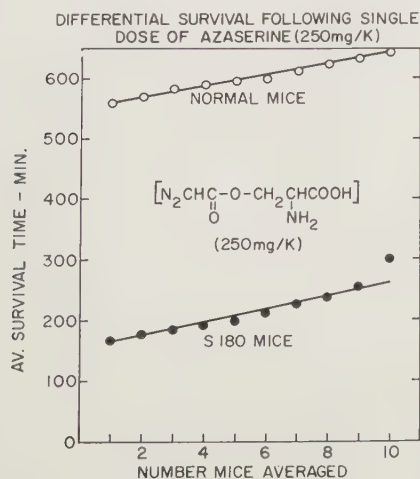


FIG. 7. Increased toxicity of Azaserine in Sarcoma 180-bearing mice compared with normal controls.

fect of the challenging compound. This is in sharp contrast to the finding with an analog of Azaserine, 6-diazo-5-oxo-*L*-norleucine (DON). The molecular difference between these two compounds resides in the substitution of a CH_2 for an oxygen atom, which change is sufficient to invert the influence of the tumor on the relative toxicity of the two compounds. Fig. 8 indicates the magnitude of the difference in response between control and experimental mice when injected with 400 mg/kg of DON and shows the protective effect provided by transplanted Sarcoma 180 tumors having an average weight of 1 g.

Discussion. The exact mechanism of the protection, or liability, which the tumor provides the host in the various circumstances cited above is uncertain. Some of the possi-

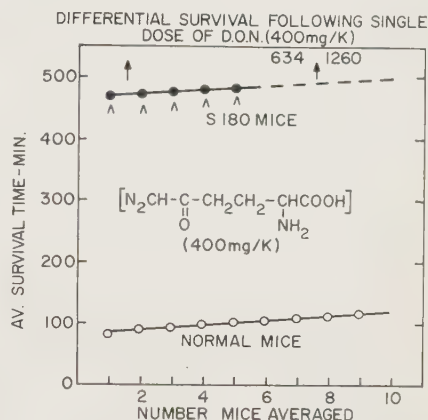


FIG. 8. Protective effect of Sarcoma 180 against toxicity of 6-diazo-5-oxo-*L*-norleucine (DON).

bilities are as follows: 1. The toxic compound targets on the tumor and is physically adsorbed, chemically bound, altered, or otherwise detoxified or enhanced at the tumor site. 2. The injected compound is detoxified or its toxicity enhanced in the peripheral blood stream by specific tumor metabolic products present only in the cancerous animal. 3. There is a differential presence or absence of specific enzymes in the tumor-bearing animal, as compared with the normal controls, capable of altering or degrading the injected compound to a more or to a less toxic form.

In experimental data in Table I it is seen that the average total mouse weight is approximately equal in the experimental and

control animals (20.6 and 21.8 g). When the tumors were removed, however, the average carcass weight of the experimental group was reduced to 16.3 g. Since the drug dose was based on total weight, the compound concentration would be expected to be equal in the carcasses of the 2 groups if the relative uptake by tumor and carcass were the same. A selective uptake or rejection of the drug by the tumor, however, would modify its concentration in the more vital carcass portion of the animal and thus, in turn, influence the survival span of the organism. The above experiments do not establish the site at which the injected compound is acted upon, but the data of Fig. 2 and 3 demonstrate that the tumor-bearing animals survived longer—as though the tumor, or its products in the peripheral blood, were selectively adsorbing or detoxifying the injected compound and thus protecting the tumor-bearing carcass at the expense of the tumor.

A similar interpretation can be made for the Sarcoma 180 experiments illustrated in Fig. 7 and 8 where diametrically opposite results were obtained with Azaserine and DON. This is an example, however, where the intervention of a tumor enzyme could be involved since it has been reported that Sarcoma 180 has an enzyme that can destroy Azaserine but not DON(9). If such an enzymic destruction of Azaserine resulted in a more toxic product for the host, it could account for the striking increase in vulnerability of the tumor-bearing animals when challenged with this compound as compared with DON. Other possibilities must also be considered, such as liver dysfunction, which may occur in Sarcoma 180-bearing mice. Since the liver sometimes becomes a detoxifying site for certain substances, a differential survival such as that observed with Azaserine (Fig. 7) could be due to an inability of the liver to detoxify the compound. In such instances the liability effect of the tumor might be an indirect one. Differential uptake between DON and Azaserine in Sarcoma 180 does not seem to be the explanation since uptake studies with ascites tumor cells showed no difference, though the tumor and the liver concentrated these compounds substantially more than did

skeletal muscle(10).

The findings reported here, together with other unpublished data, indicate that the notion that tumor-bearing animals are more vulnerable to chemical challenge because they are in a "weakened" condition is an incomplete concept and may lead to errors in the determination of maximum tolerable doses. The experiments illustrated in Fig. 7 and 8, for example, suggest that the maximum acute tolerable dose of Azaserine determined in normal, non-tumor-bearing mice would be too high for mice bearing Sarcoma 180 and, on the contrary, would be too low if the determination were for DON. In the case of PPDA and the S91 melanoma the presence of a medium-sized tumor neutralizes or detoxifies as much as one-fourth of the dose when it is administered at 100 mg/kg. That is, in a comparative experiment, the tumor-bearing animals survived as long as the normal controls which received only 75 mg/kg. Although these experiments were performed with high doses for analytical convenience, similar phenomena can be obtained at lower doses, in the vicinity of the LD₅₀, and the differences between tumor-bearing and normal animals can be measured on a percentage survival basis.

Most investigators would probably agree that cancer chemotherapy involves the successful exploitation of critical differences between normal and neoplastic tissues. The chronological phases in a systematic rational approach would appear to be detection, elucidation, and utilization of such differences. The procedures briefly reported here are capable in their present form of detecting certain subtle distinctions of a biochemical nature between various tumor-bearing animals and their normal counterparts. Upon extension, these procedures may also be helpful in elucidating those distinctions. Empirically, the mere finding of a differential response is an adequate basis for undertaking chemotherapeutic studies, whatever the explanation or mechanism of that response. However, for purposes of experimental design, a special assumption may be derived from these findings, namely, that certain specific tumor products are expressions of a metabolic distinction of

particular types of neoplastic cells and that such endogenous components could constitute a lethal accumulation for the malignant tissue if ignited by the proper reactant.

Summary. A simple experimental procedure is reported which can demonstrate a significant difference in response between certain tumor-bearing mice and their normal controls. The procedure involves challenging the two groups of animals with a lethal dose of a compound capable of reacting with the tumor or one of its metabolites. Either the cancerous or the normal animals may survive longer, depending upon both the type of tumor and the molecular configuration of the compound utilized. The difference in survival time between the tumor-bearing and normal animals is thought to be due to a modification of the toxicity of the administered compound following reaction with a tumor component. The S91 mouse melanoma

was employed as the primary model since some of the chemistry of its metabolites is known. Implications of possible utility in the study of the biochemistry of tumors and in chemotherapy are discussed.

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Renal Clearance of Ribonuclease. (23679)

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Rabinovitch and Dohi have reported an increase in the serum concentration of ribonuclease (RNAase) in bilaterally nephrectomized dogs(1). Using a new simple assay procedure for determination of RNAase in serum and urine(2), we have measured its renal clearance in normal subjects, including 5 humans and 3 dogs. In addition, observations have been made on the arteriovenous concentrations of RNAase across the intact canine kidney, as well as the changes in serum RNAase concentration following bilateral ureteral ligation.

Materials and methods. Crystalline pancreatic RNAase was obtained from Armour and Co. The activity of the enzyme was assayed upon yeast ribonucleic acid (RNA) from the California Foundation for Biochemical Research. Serum levels of RNAase were determined by diluting 0.1 ml of serum to 10

ml with a solution containing 0.02M Na_2HPO_4 and 0.14M NaCl. 0.5 ml of this solution was added to a similar volume of saline-phosphate containing 1 mg of RNA per ml. The pH remained constant at 7.8 during subsequent incubation of this mixture at 37°C for 30 minutes. After this period, 5 ml of 0.1M acetate buffer, pH 3.9, containing 3 mg/ml of bovine serum albumin (Fraction V, Armour) and 1 mg/ml of Knox gelatin was added to each mixture. The absorbence of the turbid colloid resulting from the interaction of the polymerized RNA with the acidified serum albumin was determined in a Coleman Jr. spectrophotometer at 400 m μ . The optical density so determined was translated by a standard curve into $\mu\text{g/ml}$ of polymerized RNA remaining. The difference between this value and the initial 500 μg was expressed as μg of substrate depolymerized.

TABLE I. Ribonuclease Clearances in 5 Normal Humans.

| Urine conc. ($\mu\text{g/ml}$) | Urine flow (ml/min.) | Venous conc. ($\mu\text{g/ml}$) | Clearance (ml/min.) |
|-------------------------------------|-------------------------|--------------------------------------|------------------------|
| 1.60 | 1.1 | .40 | 4.4 |
| .48 | 3.3 | .40 | 4.0 |
| .28 | 3.7 | .32 | 3.2 |
| .21 | 8.0 | .32 | 5.2 |
| .36 | 5.3 | .40 | 4.8 |
| .30 | 11.0 | .44 | 7.5 |
| .44 | 5.6 | .40 | 6.2 |
| .30 | 9.5 | .40 | 7.1 |
| .20 | 7.3 | .36 | 4.1 |
| .22 | 13.5 | .32 | 9.2 |

By comparison to a standard curve, using crystallized pancreatic RNAase, the units were translated into μg of enzyme per ml of serum. Care must be taken to avoid contamination with heparin, since this polyanion behaves as a competitive inhibitor of RNAase. Ribonuclease clearances were measured in 5 healthy male humans. Each was in a fasting state but had received an oral water load prior to and during the experiment. Urine was collected without catheterization during two 60-minute periods with a sample of venous blood at the midpoint of each period. Simultaneous endogenous creatinine clearances were used as approximations of the glomerular filtration rate. All subjects remained ambulatory throughout. Ribonuclease clearances were measured in 3 female mongrel dogs, anesthetized with sodium pentobarbital. Indwelling catheters and bladder washouts were used for the collection of urine. Each animal was hydrated with 400-700 ml of normal saline intravenously at the beginning of the experiment. Following a priming dose of creatinine, the infusing fluid was changed to creatinine in normal saline. After a suitable period of equilibration, urine was collected for 3 10-minute periods with an arterial blood sample taken at the midpoint of each period. Each dog was then given various sized priming doses of RNAase intravenously and the sustaining infusions were changed to RNAase-creatinine in normal saline. One to three clearance periods were performed at each new level of enzyme. Infusion rates were regulated by gravity drip. The clearances of exogenous creatinine were used as approxima-

tions of the glomerular filtration rate. All urine flow rates shown include washout volumes. RNAase arteriovenous differences across the kidney were measured in 3 dogs, anesthetized with sodium pentobarbital. Direct punctures of a femoral artery and right and left renal veins were used to obtain samples. This procedure was repeated after the intravenous injection of large amounts of RNAase.

Four female mongrel dogs were subjected to bilateral ureteral ligation. The animals were deprived of food and water until death on the 4th or 5th day. Daily measurements were made of serum RNAase and urea nitrogen concentrations.

Results. In presentation of experimental results in Tables I and II, no correction for body surface area has been made. In the human subjects (Table I) the mean serum RNAase concentration was $0.38 \mu\text{g/ml}$ with a range of 0.32-0.44. The mean RNAase clearance was 5.6 ml/min. with a range of 3.2-9.2. These clearances represented about 4% of the simultaneously measured glomerular filtration rate and appeared independent of variations in urine flows, from 1.1-13.5 ml/min.

The animal data showed a mean serum RNAase concentration of $0.35 \mu\text{g/ml}$ (0.18-0.50). RNAase clearances, prior to loading, averaged 3.4 ml/min. (1.5-6.1) and varied from 3-11% of the glomerular filtration rate. The clearance values for any one dog did not change significantly during loading even with RNAase serum levels acutely raised as much as 12-fold. Filtration rates remained essentially constant throughout.

Femoral artery and renal vein concentrations of RNAase were essentially the same ($A/V = 1.02$) both at normal serum levels and after a 10-fold increase.

Serum RNAase concentrations were significantly elevated in 3 of the 4 anuric dogs by the second post-operative day. However, they did not continue to rise and bore no quantitative relationship to the level of blood urea nitrogen.

Discussion. Interpretation of clearance data can be made with certainty only when certain characteristics of the test substance

TABLE II. Dog Ribonuclease Clearances at Various Serum Concentrations.

| Dosage | Urine conc. ($\mu\text{g/ml}$) | Urine flow & washout (ml/min.) | Arterial conc. ($\mu\text{g/ml}$) | Clearance (ml/min.) |
|---|-------------------------------------|--------------------------------------|--|------------------------|
| Dog 1 (10 kg) | .5 | 3.8 | .37 | 5.9 |
| | .36 | 6.3 | .37 | 6.1 |
| | .36 | 5.6 | .37 | 5.5 |
| 20 mg primer and 6 $\mu\text{g/min.}$ infusion | 1.12 | 2.7 | .50 | 6.1 |
| | 1.60 | 2.0 | .48 | 6.7 |
| | 1.60 | 2.1 | .48 | 7.0 |
| 12 $\mu\text{g/min.}$ infusion | .90 | 4.5 | .64 | 6.3 |
| | 1.04 | 3.9 | .62 | 6.6 |
| | 1.30 | 3.0 | .64 | 5.1 |
| Dog 2 (12.7 kg) | .50 | 2.2 | .50 | 2.2 |
| | .25 | 3.3 | .50 | 1.7 |
| | .23 | 3.2 | .50 | 1.5 |
| 60 mg primer and 24 $\mu\text{g/min.}$ infusion | 6.2 | 3.2 | 6.0 | 3.3 |
| | 3.6 | 3.3 | 4.8 | 2.5 |
| Dog 3 (29.1 kg) | .154 | 3.8 | .18 | 3.2 |
| | .178 | 2.4 | .16 | 2.7 |
| | .160 | 1.7 | .16 | 1.7 |
| 60 mg primer and 12 $\mu\text{g/min.}$ infusion | 1.90 | 2.1 | 1.6 | 2.5 |

are known. These include the degree of *in vivo* serum protein binding, the molecular size (which for this enzyme is about 13,750), the occurrence of metabolism and/or storage within the kidney. In the absence of any precise knowledge about some of these points, explanation of the present data is necessarily tentative. The failure of the RNAase clearance to change during loading suggests that the filtered load is neither reabsorbed nor added to by the tubules. The small magnitude of the clearance relative to the filtration rate may reflect a high degree of protein binding, with consequent inability of a large portion of serum RNAase to pass the glomerular membrane. A recent paper by McGeachin and Hargan(3) proposes just such a mechanism of excretion for another enzyme, serum amylase. An alternative explanation for our data is that the filtered load of RNAase is normally at or above its tubular maximum.

The arterio-venous concentrations of RNAase across the kidney suggest that this organ neither metabolizes nor produces significant amounts of RNAase under the conditions of experiment.

Serum RNAase concentrations are elevated in anuric dogs as reported by Rabinovitch and Dohi(1) but the levels cannot be used either as an index of catabolic rate or duration of anuria.

Summary. (1) The magnitude of the renal clearance of RNAase and its failure to change during loading suggests filtration of an unbound fraction of enzyme as the mode of excretion. (2) The arterial and renal vein concentrations of RNAase across the kidney were identical under the conditions of these experiments. (3) RNAase serum levels were elevated in 3 of 4 anuric dogs but did not continue to rise with blood urea nitrogen.

The authors wish to gratefully acknowledge the advice of Dr. George Schreiner of this hospital.

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Effect of Carbon Tetrachloride Inhalation on Rat Serum Enzymes. (23680)

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Ball and Kay(1) showed that exposure of rats to carbon tetrachloride vapor results in a decrease in serum esterase activity. Subcutaneous injection of carbon tetrachloride into rats was found by Affonso *et al.*(2) to produce an elevation in serum xanthine oxidase activity. Increases in serum glutamic-oxaloacetic-transaminase (GOT) have been reported in humans following severe accidental exposure to carbon tetrachloride(3).

Since these investigations were carried out under widely varying conditions, it seemed advisable to study these 3 enzymes simultaneously under controlled conditions of exposure to carbon tetrachloride vapor. This preliminary report deals with the effect of acute inhalation of varying concentrations of carbon tetrachloride on rat serum esterase, xanthine oxidase, and glutamic-oxaloacetic-transaminase (GOT).

Methods. Male rats of the Sprague-Dawley strain, 150-200 g in weight, were used in this investigation. The animals were maintained on an *ad libitum* diet of Purina Laboratory Chow. The animals were exposed to known concentrations of carbon tetrachloride vapor using a dynamic exposure chamber, according to the procedure described by Ball and Kay (1). Chamber concentrations of carbon tetrachloride were checked during the course of the experiment by a modification of the method of Peterson *et al.*(4), and were found to be within $\pm 10\%$ of calculated values. Three concentrations were selected for study: 40 rats were exposed to 1500 ppm of CCl_4 vapor, 40 rats to 1000 ppm, and 40 rats to 250 ppm. In this series of acute experiments, exposure was limited to a single 4 hour period. Immediately following the exposure, 4 rats in each group were killed by exsanguination, and enzyme activities determined on the serum from each animal. On each subsequent day for a period of 9 days, 4 additional animals from each group were sacrificed. Thirty unexposed rats (150-200 g in weight) were used

for the determination of control levels of these enzymes in rat serum. Serum esterase activity was determined by the Gomori colorimetric technic(5), xanthine oxidase activity by the method of Westerfeld *et al.*(6), and glutamic-oxaloacetic-transaminase (GOT) activity by the procedure of Steinberg *et al.*(7).

Results. Control values and the standard deviations for the activity of these enzyme systems in rat serum were: for transaminase 171 ± 41 units/ml serum at 25°C ; for xanthine oxidase, 191 ± 45 mg xanthine/ml serum/hr; and for esterase, 8.0 ± 1.8 mg phenol/ml serum/hr.

Fig. 1A shows the changes in serum enzyme activity resulting from exposure of the rats to 1500 ppm of carbon tetrachloride vapor. In this figure, the control value is taken as 100%. Changes in enzyme activity resulting from the exposure are expressed as percentage of the control value. It can be seen from Fig. 1A that the most striking change occurred in serum GOT activity. In 24 hours, the activity of this enzyme was 765% of control levels. On the second day following exposure, GOT activity was 570% of control values. By the fourth day this value had returned to the control level.

Xanthine oxidase activity (Fig. 1A) was also increased as a result of exposure to 1500 ppm of carbon tetrachloride. This effect is greatest on the second day following exposure, when activity was 265% of control levels. Xanthine oxidase levels had returned to control values within 4 days following the exposure.

Serum esterase activity (Fig. 1A) decreased as a result of the 4 hr exposure to carbon tetrachloride. On the second day following exposure, serum esterase values were only 54% of control levels. Within 4 days, this value had returned to essentially the control level.

Fig. 1B shows the results obtained after the exposure of rats to 1000 ppm of carbon tetrachloride. Changes in enzymatic activity were

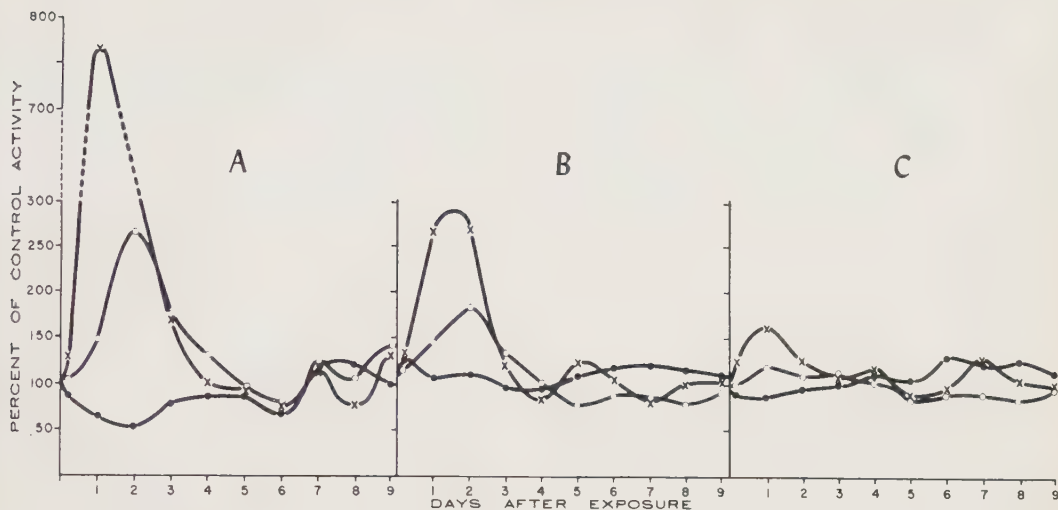


FIG. 1. The effect of a single 4-hour inhalation of carbon tetrachloride vapor on rat serum esterase (●—●), xanthine oxidase (○—○), and glutamic oxaloacetic-transaminase (GOT) (×—×). 1500 ppm in air (A), 1000 ppm in air (B), 250 ppm in air (C).

less marked at this concentration than at 1500 ppm. Serum GOT reached its peak activity (288% of normal) on the second day following exposure. By the third day this value had returned to control levels. Xanthine oxidase activity rose to 186% of control levels on the second day following exposure and returned to control levels by the fourth day. Serum esterase activity was apparently unaffected by a single 4 hour exposure at this concentration.

Fig. 1C shows the results obtained after a 4 hr exposure of rats to 250 ppm of carbon tetrachloride. The only enzyme significantly affected by this concentration of vapor was serum GOT, whose activity rose to 163% of control levels within 24 hours. Serum xanthine oxidase and esterase activities were essentially unaltered following this level of exposure.

Discussion. Exposure of male rats to 1500 ppm of carbon tetrachloride vapor brought about increases in serum glutamic-oxaloacetic-transaminase (GOT) and xanthine oxidase activities and a decrease in serum esterase activity. Serum GOT appears to be the most sensitive in its response, showing a marked rise in activity following a 4 hr exposure of rats to 250 ppm of carbon tetrachloride vapor. These studies are based on a single acute exposure and do not necessarily reflect the response to chronic exposure at low vapor con-

centrations. Ball and Kay(1) found that lowered serum esterase activities tended to return to control levels even though the animals were exposed daily to low concentrations of carbon tetrachloride vapor. The effect of repeated low concentrations of carbon tetrachloride vapor on serum GOT and xanthine oxidase activity will be reported later.

It remains to be demonstrated whether any or all of these enzymatic changes are directly related to the production of liver damage. Bruns and Neuhaus(8) have shown that, in mice, following the intraperitoneal injection of carbon tetrachloride, increases in serum aldolase and phosphohexose isomerase activity parallel decreases in the activity of these enzymes in the liver. The authors(8) suggest that injured liver cells may lose enzymes to the serum. Affonso *et al.*(2) found an increased xanthine oxidase activity in both liver and serum following the subcutaneous injection of carbon tetrachloride into rats. These workers(2) postulated that this increase might be due to the release of enzyme from lipoprotein.

Although either hypothesis could explain the increased activity of serum GOT and xanthine oxidase following carbon tetrachloride exposure, the decrease in serum esterase activity cannot be attributed to either of these mechanisms.

The practical advantage of serum enzyme

determinations as a sensitive measure of the biological response to inhalation of toxic vapors encourages further investigation into the nature, extent, and significance of these enzyme changes.

Summary. Serum esterase, xanthine oxidase, and glutamic-oxaloacetic-transaminase (GOT) activities were followed for 9 days after a single 4 hr exposure of male rats to carbon tetrachloride vapors. 1500 ppm of carbon tetrachloride in air produced marked changes in all 3 enzyme systems. 1000 ppm affected only serum GOT and xanthine oxidase activities. At 250 ppm, only serum GOT activity was significantly affected.

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Bentonite Flocculation Test for Rheumatoid Arthritis.* (23681)

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The various serological tests for rheumatoid arthritis have attracted increasing attention and interest in recent years, largely because they have been widely recognized as useful diagnostic measures. As an objective diagnostic criterion, the test is helpful in segregating an homogeneous type of rheumatoid arthritis from a general and heterogeneous group of polyarthritides. In addition, the test makes it possible for workers in different parts of the world to agree on the definition of this disease entity. Some basis for such agreement is especially important in determining the prevalence and incidence of rheumatoid arthritis in diverse and widely separated populations and also in critically evaluating therapeutic measures used in the management of this disease. A variety of serological tests for rheumatoid arthritis have been described since the first one was reported(1). A comprehensive review of the methods, principles, relative merits, and clinical correlation of the different

tests used to date has been published recently by Ziff(2). All serological tests for rheumatoid arthritis depend on clumping of particulate matter by addition of serum from patients. Some of the tests employ sheep cells; in this type of test, the rbc are either mixed with a subagglutinating amount of immune rabbit serum or with human gamma globulin (HGG); later several dilutions of patient's serum are added to rbc. Other tests used colloidion or latex particles coated with HGG. In the present study, bentonite particles were coated with HGG. These particles were employed by Bozicevich *et al.*(3) to adsorb antigenic material from trichina in a serologic test for the presence of antibodies in the serum of patients infected with trichina. The technic used in the present study is a slight modification of that published in 1951. Although useful, most of these tests are complicated, time consuming, and require specialized technics. The bentonite flocculation test, described here, is simple and can be done in 20 minutes once the stock solutions are prepared.

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** National Institute of Arthritis and Metabolic Diseases.

Bentonite, a colloidal clay of powder consistency, is stable and readily available. It consists of abundant, negatively charged particles whose surface/mass ratio is unusually large. After the particles are "sensitized" with normal human serum gamma globulin (Fraction II), they clump when mixed with serum from patients with rheumatoid arthritis.

Preparation of bentonite suspension. A convenient way to prepare bentonite suspension is as follows: Suspend 0.5 g of bentonite in 100 ml of distilled water and homogenize it with the aid of a blender. The bentonite suspension is placed in a glass-stoppered graduate and diluted with distilled water to a volume of 500 ml. After shaking the suspension and allowing it to settle for one hour, the supernate is removed and placed in 90 ml centrifuge tubes. The tubes are centrifuged (by tachometer) at 1,300 rpm for 15 minutes, using an International Centrifuge, size 2, with No. 240 head. The supernates are collected and centrifuged at 1,600 rpm for 15 minutes. The accumulated sediment is suspended in 100 ml distilled water with the aid of a blender. The bentonite suspension may be kept at room temperature for several months, and it should be shaken before removing an aliquot. This technic yields a range of particle size which does not tend to give false positive or false negative reactions.

Preparation of F II solution. One-half g of lyophilized Fraction II[†] (gamma globulin) derived from pooled normal human sera is dissolved in 50 ml veronal buffer. The veronal buffer is made by dissolving 0.184 g barbituric acid + 1.03 g sodium barbiturate in 100 ml distilled water. This buffer has 0.06 molarity, and its pH is 8.6. The F II solution may be stored at 5°C for one or 2 months. It may be clarified by centrifugation.

Preparation of sensitized bentonite particles. Ten ml of the bentonite suspension is centrifuged and the sediment taken up in 1 ml of distilled water. To this suspension, 2 ml of

the F II solution is added and the 2 components gently mixed by tilting and rotating. The suspension is then allowed to stand for 15 to 30 minutes at room temperature to permit adsorption, 15 ml of distilled water is added, and the suspension is gently mixed again. The suspension is centrifuged, and the sediment suspended in 15 ml of water and vigorously shaken for 30 seconds to disperse the particles. This suspension is centrifuged again, and the sediment is taken up in 5 ml water. One ml of 0.1% methylene blue solution is added to the suspension and again vigorously shaken. After 5 minutes, 10 ml of water are added, the suspension is centrifuged, and the sediment is taken up in 10 ml of distilled water. Washing is repeated and the sediment taken up in 4 ml of 0.05 molar phosphate buffer of pH 7.3. It is desirable to add 0.1 ml of 1% Tween 80 to this sensitized bentonite suspension. The serum[‡] to be tested is heated for 30 minutes at 56°C prior to use. Microscopic slides (3" x 2") with 12 rings, designed for serological flocculation tests, are used to carry out the test. Into each ring on the slide is placed 0.1 ml of serial twofold dilutions of serum in saline. One drop (about 0.025 ml) of sensitized bentonite suspension is added to each ring. (For this purpose, it is convenient to use a capillary pipette which will deliver about 40 drops per ml.) The slide is then placed on a Boerner-type rotating machine and rotated 100 to 120 times per minute for 20 minutes, and read immediately under a microscope at low-power magnification (60 X). Drying is to be avoided. In a 4-plus reaction, all of the sensitized particles are clumped in separate masses. There may be a few large or a number of small clumps, depending upon the titer of the serum. Nevertheless, the fields between the flocs are almost clear. In a 3+ reaction, three-fourths of the bentonite particles are clumped; in a 2+ reaction, half; and in a 1+ reaction, one-fourth. The remaining particles are fairly evenly dispersed. In a negative test, the sensitized bentonite particles remain in suspension. The flocculation test

[†] Fraction II is obtained by fractional precipitation of pooled normal human serum by the method described by E. J. Cohn, *et al.*, *J. Am. Chem. Soc.*, 1950, v72, 465.

[‡] Freshly collected serum is preferable. Serum stored at 5°C for as long as 2 weeks has been found satisfactory.

TABLE I. Bentonite Flocculation Test in Rheumatoid Arthritis (41 Cases).

| Age and sex | Duration of arthritis (yr) | Subcut. nodules | ESR (mm/hr) | Titer (flocculation of 2+ or greater) |
|-------------|----------------------------|-----------------|-------------|---------------------------------------|
| 21 ♀ | 2½ | No | 57 (WS)* | 8192 |
| 43 ♀ | 6 | Yes | 127 " | >4096 |
| 55 ♀ | 4 | No | 104 " | 2048 |
| 52 ♂ | 2 | Yes | 40 " | " |
| 37 ♀ | 8 | No | 37 " | " |
| 31 ♀ | ½ | Yes | 60 " | 1024 |
| 35 ♀ | 6 | " | 59 " | >1024 |
| 37 ♂ | 7 | No | 38 " | 1024 |
| 65 ♀ | 8 | " | 93 " | 256 |
| 51 ♂ | 2 | Yes | 74 " | " |
| 47 ♂ | 6 | " | 43 " | " |
| 32 ♀ | 7 | No | 41 (WN)* | " |
| 34 ♂ | 10 | Yes | 36 " | " |
| 66 ♀ | 39 | " | 36 " | " |
| 51 ♀ | 10 | " | 28 " | " |
| 40 ♂ | 14 | No | 25 " | " |
| 44 ♀ | 13 | Yes | 16 (WS) | " |
| 18 ♂ | 5 | No | 6 " | " |
| 52 ♂ | 10 | Yes | 97 " | >128 |
| 61 ♂ | 19 | No | 38 (WN) | 128 |
| 46 ♂ | 15 | " | 34 " | " |
| 39 ♂ | 14 | Yes | 32 " | " |
| 41 ♂ | 2 | No | 82 (WS) | 64 |
| 40 ♂ | 6 | Yes | 60 " | " |
| 21 ♀ | 1 | No | 57 " | " |
| 30 ♀ | 4 | " | 54 " | " |
| 63 ♂ | 4 | Yes | 36 (WN) | " |
| 35 ♂ | 8 | No | 32 " | " |
| 60 ♀ | 3 | " | 45 " | 32 |
| 48 ♂ | 1 | " | 30 " | " |
| 68 ♂ | 12 | " | 23 " | " |
| 54 ♂ | 13 | Yes | 6 " | " |
| 56 ♂† | 12 | " | 105 (WS) | 0 |
| 31 ♀ | 2 | No | 77 " | 0 |
| 34 ♀† | 13 | " | 40 " | 0 |
| 53 ♂† | 14 | " | 38 (WN) | 0 |
| 69 ♀† | 11 | " | 30 (WS) | 0 |
| 34 ♂ | 7 | " | 27 (WN) | 0 |
| 57 ♀† | 8 | Yes | 10 " | 0 |
| 25 ♂† | ½ | " | 2 (WS) | 0 |
| 57 ♀ | 7 | No | " | 0 |

* WS = Westergren; WN = Wintrobe corrected.

† Sera from these 6 patients gave negative sheep cell agglutination test, as well as negative BFT. Sera from the remaining 3 patients whose BFT were negative were not tested for sheep cell agglutinins.

is considered positive when a 2+ or stronger clumping occurs in a serum dilution of 1 to 32 or higher. Although a negative serum does not cause any clumping in dilutions up to about 1 to 1,000, occasionally a 1+ or 2+ flocculation may occur in dilutions of 1 to 1,000 or higher. With each test, serial saline dilutions of a known positive and a known negative serum should be included in order to de-

tect any change in the sensitized bentonite particles. Debris in the sensitized bentonite mixture or the control negative serum, may cause clumping of the particles. With experience, the spurious aggregates can readily be distinguished from true flocculation. When the test is positive, the flocs are of uniform density, evenly distributed in the microscopic field and free of "hyaline-like" material or other debris.

Results. In patients with unequivocal rheumatoid arthritis. Sera from 41 adult patients with unquestionable rheumatoid arthritis have been tested. The pertinent clinical and laboratory data and results of the test are listed in Table I. In 32 patients (78%), the tests were positive. None of the patients had ankylosing spondylitis or psoriasis. The BFT in 9 patients with rheumatoid arthritis was negative. The sera of 6 of these patients (including 3 with subcutaneous nodules) were also tested for sensitized sheep erythrocyte agglutination reaction (SSEA) using the euglobulin fraction and all 6 were negative.

In control subjects. Sera from 163 control subjects were tested (Table II). Normal subjects, patients with arthritis and rheumatic diseases other than rheumatoid arthritis, as well as patients with many unrelated diseases were included. None of the sera from the 15 normal persons gave a positive test. Of the sera from 148 control patients, 3 (2%) reacted positively. It is of interest that one of these 3 was from a patient with systemic lupus erythematosus and another from a patient with macroglobulinemia. Of the 160 sera that were negative, 155 failed to show any flocculation at all, even in the first dilution (1:4); 4 sera caused flocculation in dilutions of 1:4 or 1:8; and one, in dilution up to 1:16. None of these 5 sera gave a 4-plus flocculation in any dilution or a 3-plus flocculation in dilutions of 1:8 or higher.

Comparison of BFT and sensitized sheep erythrocyte agglutination (SSEA) test on sera from same patients. A series of 64 sera from 64 patients was subjected to both tests. The SSEA tests were done in the laboratory of Dr. R. R. Williams, Jr., at the Nat. Inst. of Arthritis and Metabolic Diseases by the method described by Ziff and his associates(4).

TABLE II. 163 Cases Included in Control Series.*

| | |
|--|-------|
| Rheumatic diseases other than rheumatoid arthritis | 28 |
| Rheumatic heart disease (active and inactive) | 10 |
| Gout | 9 |
| Reiter's | 3 |
| Scleroderma | 2 |
| Dermatomyositis | 2 |
| Systemic lupus erythematosus | 1 (1) |
| Rheumatic fever | 1 |
| Normal subjects | 15 |
| Carcinoma of various organs | 41 |
| Hypercholesterolemia | 5 |
| Uveitis, brain tumor, and macroglobulinemia | 5 (1) |
| Multiple myeloma | 4 |
| Congenital heart disease | 4 |
| Undiagnosed disease | 4 |
| Muscular dystrophy | 4 |
| Osteoporosis | 3 |
| Mental defective | 3 |
| Stomatitis | 3 |
| Arteriosclerotic heart disease | 3 |
| Acute leukemia | 3 (1) |
| Diabetes mellitus | 2 |
| Pseudohemaphroditism | 2 |
| Actinomycosis | 2 |
| Viral infections | 3 |
| Hypothyroidism | 2 |
| Miscellaneous | 27 |

One case of each of the following: Blastomycosis, cerebral degeneration, cirrhosis of the liver, cryoglobulinemia, cryptococcosis, emphysema, gastrointestinal bleeding, hepatitis, hepatosplenomegaly of unknown causes, histoplasmosis, Hodgkin's disease, Huntington's chorea, hyperparathyroidism, hypertensive heart disease, hyperthyroidism, hyperuricemia (without gout), hypoproteinemia, lymphoma, nephrosis, ovarian agenesis, Paget's disease, persistent lactation, sexual precocity, sprue, thrombocytopenia, tuberculous lymphadenitis, and Wilson's disease.

* No. in parentheses indicates the 3 false positive reactions. The test was negative in all other control cases.

The sera for the latter test were separated from the clot within hours after collection and frozen. Several days later, they were thawed, heated at 56°C for 30 minutes, absorbed with sheep red blood cells, and fractionated. The euglobulin fraction was either tested the next

TABLE III. Comparison of BFT and SSEA Tests in 64 Sera.

| BFT+ SSEA+ | BFT- SSEA- | BFT- SSEA+ | BFT+ SSEA- |
|---------------|---------------|---------------|---------------|
| 23 | 40 | 1 | 0 |

day or frozen and tested days later. The BFT was done on whole, unabsorbed, inactivated sera separated from the clot soon after collection and refrigerated but not frozen. The results are recorded in Table III. In both positive and negative reacting sera, there was agreement between both tests in 63 samples (98%) and discordant results in one (Table III). The titers of sera that gave positive reactions were often higher in the SSEA test than in the BFT. Comparison of the BFT and the Latex test was not possible because Latex particles were not available to the authors.

Summary. 1) When bentonite particles of a selected size are "sensitized" by human gamma globulin (Fraction II) and then mixed with serum from patients with rheumatoid arthritis, they flocculate. This reaction was observed in 78% of adult patients with definite rheumatoid arthritis, in 2% of control patients, and in none of a group of normal subjects. Sera tested by the SSEA test and the BFT showed agreement in 98%. 2) Procedures for doing the BFT are described. The test is simple and rapid. The sera to be tested are not absorbed or fractionated.

We wish to acknowledge with gratitude the cooperation of Dr. Ruth Wichelhausen of Mt. Alto Veterans Admin. Hospital, Washington, D. C., and Dr. Herbert Kayden of Goldwater Memorial Hospital, New York, in permitting us to examine patients and collect blood samples used.

Addendum: The series of cases tested has increased since this paper was submitted for publication. It now includes a total of 82 verified cases of rheumatoid arthritis in adults in which the bentonite flocculation test was positive in 85.5% and 227 controls in which the test was negative in 97.4%.

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Urinary Excretion of Uric Acid and Allantoin by the X-Irradiated-Adrenalectomized Rat.*† (23682)

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In a study of purine excretion by the whole-body X-irradiated dog(1) it was observed that an increased excretion of uric acid and allantoin occurred which was similar to that reported by Fajans *et al.*(2) after injection of the dog with adrenocorticotrophic hormone. Because increased adrenal activity has been reported to occur a few hours after irradiation in both the mouse(3) and monkey(4), it would appear that increased adrenal activity following irradiation may stimulate increased purine excretion. It is of interest, therefore, to study the role of the adrenal gland in relation to the post-irradiation increases of urinary purine catabolite excretion which have been observed in man(5), dog(1,6), rabbit(7), and the rat(8,9).

The present communication is concerned with an attempt to elucidate the contribution by the adrenal gland to the post-irradiation rise of urinary purine catabolite excretion in the rat.

Methods. Female Sprague-Dawley rats were fasted for 24 hours before irradiation and for the 24-hour post-irradiation period. Body weights (before fasting) ranged between 170 and 190 g in the first experiment (Fig. 1) and between 180 and 210 g in the second experiment (Tables I and II). Each rat irradiated, received a whole-body dose of 250 KVP X-ray (15 ma; 0.5 mm Cu, 1 mm Al filter) at a dose rate of 26 r per minute with a tar-

get-to-midline distance of 40 inches. During irradiation the rats were held in sector-shaped acetate-plastic containers which were rotated under the X-ray beam. The rat containers were tilted at a 19° angle resulting in essentially a constant dose rate through the midline of the rats. Control rats were placed in identical plastic containers, but were not irradiated. Following irradiation the animals were maintained in individual metabolism cages for 24 hours and 24-hour urine samples were collected as described by Kay *et al.*(10). Urine specimens were stored under refrigeration. Bilateral adrenalectomy and sham-operations (control animals) were performed 2 days prior to the time of irradiation. The control animals were given water *ad lib.*; the adrenalectomized animals 0.1% NaCl solution *ad lib.* Allantoin was determined by the method of Young and Conway(11) and uric acid by the uricase-spectrophotometric method of Feichtmeir and Wrenn(12).

Results. Previous reports on purine excretion by the rat following irradiation have been limited to the measurement of allantoin over a 3- to 6-hour period after a single X-ray dose of 400 r(8) and to uric acid for several days after a single X-ray dose of 550 r(9). Consequently a preliminary experiment was performed to determine the magnitude of in-

TABLE I. Urinary Uric Acid and Allantoin Excretion by Adrenalectomized Rats Irradiated with 600 r.*

| Treatment† | mg/24 hr‡ | |
|------------------------|------------|------------|
| | Uric acid | Allantoin |
| Sham operated—600 r | 2.71 (.24) | 46.0 (1.3) |
| " " — 0 r | 1.62 (.04) | 26.4 (1.3) |
| Adrenalectomized—600 r | 2.30 (.22) | 42.3 (1.3) |
| " " — 0 r | 1.10 (.04) | 25.0 (.7) |

* Body wt range 180-210 g. Urine samples were collected during first 24 hr following irradiation.

† Animals fasted for 24 hr prior to irradiation and during the urine collection period.

‡ Values in parentheses are stand. error of mean. (Four animals in each group, except the 0 r sham operated group which contained 3 animals.)

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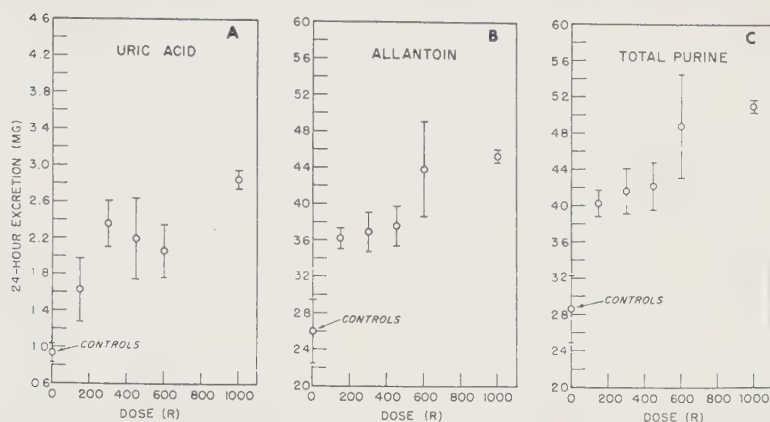


FIG. 1. Uric acid and allantoin excretion during first 24 hours following X-irradiation of rats over a dose range of 150 to 1000r. Animals were fasted for 24 hours prior to irradiation and during the urine collection. The control group (0 r) contained 4 animals, all other groups contained 3. Bars indicate standard error of means. Total purine is estimated as the sum of uric acid and allantoin (allantoin was converted to equivalent weight of uric acid).

crease in total purine excretion (as estimated by the sum of uric acid and allantoin)[†] during the first 24 hours following irradiation of intact rats with doses ranging between 150 and 1000 r (Fig. 1).

In this experiment deaths occurred only in the 1000 r group, one animal on day 5 and 2 on day 10. Uric acid excretion (Fig. 1A) was increased following irradiation with doses between 150 and 1000 r over that of the 0 r controls. Allantoin and total purine excretion as estimated by the sum of uric acid and allantoin, (Fig. 1B and 1C) were also increased after irradiation with a trend towards increased amounts with increasing dose.

To study the role of the adrenal gland with respect to post-irradiation increases in urinary purine excretion, adrenalectomized rats were subjected to 600 r whole body X-irradiation and urinary excretion of uric acid and allantoin was determined. The data (Table I) show that adrenalectomy did not prevent a rise in either uric acid or allantoin excretion during the first 24 hours post-irradiation. These results, expressed as total purine (sum of uric acid and allantoin) are presented in Table II. The statistical analysis of variance is given in the bottom half of Table II(14).

† Hunter *et al.*(13) have shown that in urine excreted by normal rats these 2 compounds account for about 97% of the purine base nitrogen plus allantoin nitrogen.

It should be noted that the interaction term is not significant, thus implying that the effects due to irradiation and adrenalectomy are independent and additive. Adrenalectomy caused a mean decrease in total purine excretion of 8% ($p < 0.025$) whereas irradiation

TABLE II. The Influence of Adrenalectomy and Irradiation on Total Urinary Purine Excretion.

| Total purines (mg/24 hr)* | | | | Increased excretion due to irradiation |
|--|--------------------|-------------|-------------|--|
| Control | Adrenalectomized | Mean | | |
| 600 r | 51.6 | 47.3 | 49.5 | 20.8 ± 1.1 |
| 0 r | 29.7 | 27.7 | 28.7 | |
| Mean | 40.7 | 37.5 | | |
| Decreased excretion due to adrenalectomy | 3.2 ± 1.1† | | | |
| Analysis of variance | | | | |
| Variation due to | Degrees of freedom | Mean square | Probability | |
| Adrenalectomy | 1 | 40 | <.025 | |
| Irradiation | 1 | 1719 | <.01 | |
| Interaction | 1 | 4 | Not signif. | |
| Experimental error | 11‡ | 5 | — | |

* Total purine excretion was calculated from data in Table I as the sum of uric acid and allantoin (expressed as equivalent wt of uric acid).

† Stand. error of mean.

‡ Degrees of freedom were reduced by one to compensate for a missing value which was estimated (due to accidental loss of one animal in the 0 r sham operated group resulting in 3 animals in this group and 4 animals in each of the other groups).

caused a mean increase in total purine excretion of 73% ($p < 0.01$).[§]

Discussion. It is evident from the data presented that X-irradiation of the rat causes a relatively large increase in the excretion of uric acid and allantoin during the first 24 hours. It is probable that this increase in total purine excretion is the result of tissue breakdown with the concomitant loss of nucleic acids(16) and other purine containing compounds(17). Lymphatic tissue breakdown following irradiation is rapid as judged by the rate of decrease in spleen and thymus weights(16,18,19) and this loss of tissue mass is accompanied by a drastic reduction in organ DNA content(16,17,18). In addition it has been shown that lymphatic tissue breakdown after X-irradiation of the rat and mouse with doses of 150 r and 200 r or greater is independent of adrenal-cortical action(20,21). These findings are consistent with the data obtained in the present experiment which show that the *magnitude of increased total purine excretion due to X-irradiation* is not reduced following bilateral adrenalectomy. Thus, although the adrenal-cortical activity influences total purine excretion to a small degree, increased purine excretion following irradiation of the rat is not dependent upon adrenal-cortical activity.

Summary. 1. X-irradiation of fasting rats with doses ranging between 150 and 1000 r caused an increased excretion of uric acid (75 to 206%) and allantoin (39 to 74%) during the first 24 hours. 2. Total purine excretion (sum of uric acid plus allantoin) tended to increase with increasing X-radiation dose. 3. Removal of adrenal glands reduced to a small extent the amount of total purine excreted but the magnitude of the increase due

only to irradiation was the same in adrenalectomized and intact rats. 4. It is concluded that increased purine excretion following irradiation of the rat is not dependent on adrenal gland activity.

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[§] Romanoff and Hunt(15) have previously reported a decreased excretion of allantoin following adrenalectomy in the rat.

Distribution of Growth Hormone Among Cell Fractions Isolated from Pituitary Gland. (23683)

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By the technic of differential centrifugation, tissue homogenates can be fractionated to obtain a rough separation of cellular elements. This technic has now been applied to pituitary homogenates, with a view to ascertaining the distribution of growth hormone (GH) among the isolated fractions, *viz.* nuclear fraction (nuclei contaminated with some cytoplasmic material), mitochondrial fraction ('large granules'), 'fluffy layer' (material overlying the sedimented mitochondria), microsomal fraction ('small granules'), and supernatant fraction ('cell sap'). Since it was essential that fresh material be used for this fractionation, the experiments were performed with freshly excised rat pituitary glands.

Materials and methods. All isolative procedures were carried out at 0-2°C. In each of the 2 experiments, the pituitary tissue (approx. 0.5 g) obtained from 100 young albino rats was homogenized in 0.25 M sucrose solution with a Potter-type homogenizer. The debris obtained on centrifugation (600 g for 10 min.) was re-homogenized and recentrifuged, and the sedimented material ('nuclear fraction') treated as described below. The combined supernatant fluids (approx. 5 ml) were centrifuged to sediment the mitochondrial fraction (12,000 g for 15 min.), from which the loose 'fluffy layer' was separated. These 2 fractions were washed once. The supernatant fluid and washings were combined, and centrifuged (20,000 g for 90 min.) to give the microsomal fraction and the supernatant fraction. Electron microscopy (kindly carried out by Mr. M. S. C. Birbeck) showed that mitochondria were present in the 'mitochondrial fraction' and absent from the 'microsomal fraction', but each fraction contained some particles which could not readily be

identified. Each fraction was dialyzed overnight against water containing a trace of ammonium carbonate, the alkaline pH thus maintained being likely to minimize inactivation of the GH. The dialyzed suspensions were finally lyophilized. The fractions were suspended in saline and injected subcutaneously into rats in eight divided doses over a period of 4 days(1). On the fifth day the rats were autopsied, the tibia preserved in 10% formalin and stained by the silver nitrate method. The tibia were measured by taking 10 measurements along the epiphyseal line to arrive at the mean width. All the fractions from each experiment were tested simultaneously. With a standard preparation of bovine GH, tested under similar conditions, a ten-fold increase in dose gave a net increase of 107 μ ($\lambda = 0.241$) in epiphyseal width; this value was used in the calculation of relative potencies. The mean epiphyseal widths in rats given the pituitary fractions were in all instances greater than those in control rats treated only with saline.

Results. Table I shows the yields of the dried fractions, and the amounts of activity in the particulate fractions relative to the amount in the supernatant fraction.

It is evident that a substantial proportion of the activity was present in the supernatant fraction, but that appreciable activity was present in each of the particulate fractions. The dry weight of the supernatant fraction being relatively high, the particulate fractions were in general richer in GH, in terms of dry weight, than the supernatant fraction. It cannot, of course, be assumed that the potency values obtained from the tibia test are an absolute index in the amount of GH present, since it is known that ACTH and thyrotrophin, which were presumably present in some of the fractions examined, may modify the effectiveness of GH in this test.

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TABLE I. Growth-Promoting Potency of Rat-Pituitary Fractions.

| Fraction | Yield of dried fraction (mg/g pituitary tissue) | | Activity of fraction, in arbitrary units (supernatant fraction = 100)* | | | |
|----------------|--|--------|---|--------|-----------------------|--------|
| | Exp. A | Exp. B | Activity, mg fraction | | Total amt of activity | |
| | | | Exp. A | Exp. B | Exp. A | Exp. B |
| 'Nuclear' | 11 | 22 | 110 | 30 | 16 | 14 |
| Mitochondrial | 17 | 35 | 270 | 55 | 62 | 39 |
| 'Fluffy layer' | 24 | 8 | 110 | 120 | 36 | 86 |
| Microsomal | 26 | 20 | 130 | 170 | 46 | 71 |
| Supernatant | 74 | 49 | (100) | (100) | (100) | (100) |

* Total amt of activity, g of original pituitary tissue was equivalent to 2.4 mg (Exp. A) or 1.5 mg (Exp. B) of bovine GH, batch 22KR2.

Ziegler and Melchior(2) have now made a similar comparison of fractions isolated from rat pituitary tissue; a high proportion of the GH activity was found in the supernatant fraction, essentially as in the present study. Assays for gonadotrophic activity, on rat-pituitary fractions analogous to those now isolated, suggest that, at least in the case of follicle-stimulating hormone, activity may not be consistently localized in a single fraction (2,3,4). However, it is difficult to reconcile the present results with those recently reported by Brown and Hess(5), who concluded after careful study that GH was located mainly in the mitochondrial fraction. It is difficult to accept the suggestion of these authors that the occurrence of high hormonal activity in the supernatant, as in the experiments of Ziegler and Melchior(2), indicates the use of severe conditions; in the present study the glands were fresh and were homogenized gently. A more probable explanation is that bovine pituitaries, as used by Brown and Hess, may differ from rat pituitaries with respect to the intracellular localization of GH, the GH in the supernatant fraction being conceivably in transit from secretory particles to the cell exterior.

Summary. Fractions isolated from rat pituitary homogenates by differential centrifugation have been assayed for growth-promoting activity by the tibia test. The supernatant fraction contained a relatively high proportion of the activity, but no sharp localization was evident.

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Milk Let-Down Activity of Synthetic Oxytocin (Syntocinon) and Relaxin in Lactating Rats.* (23684)

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A recent investigation(1) showed Nembutal was able totally to inhibit milk let-down in the lactating rat but the young were able to obtain milk when oxytocin (Armour) was injected intravenously while the offspring were actively sucking. Values thus obtained when compared with normal let-down data(2) suggested the amount of oxytocin released by lactating rats in response to nursing stimuli was in the range of less than .02 to .1 USP/kg. It occurred to us such a procedure could serve as a method of bioassay for substances possessing milk let-down activity. With this in mind, we have assayed, in the present study, a new synthetic oxytocin (Syntocinon) which has been demonstrated to possess all other properties of natural oxytocin(3-7) and relaxin which has been claimed to have milk let-down activity(8).

Materials and methods. Forty primiparous albino lactating rats weighing 280-320 g were used. Shortly after birth each litter was reduced to 6 young and when 14 days old was isolated from their mother for 10 hours. Each mother was injected with Nembutal (4.5 mg/100 g) i.p. 10-20 minutes prior to end of isolation period and when completely anaesthetized, laid on her side and her litter replaced. Thirty rats received Syntocinon[‡] in dose of .02, .05 or .1 USP/kg i.v. in a volume not exceeding .06 ml. into the superior epigastric vein a few minutes after the young had been replaced and while they were actively sucking. Ten others similarly received either 600 or 1200 Guinea Pig Units (GPU) relaxin.[§] After each litter had been nursed for 30 min-

utes, they were removed, weighed, killed by decapitation and stomach contents removed and weighed.

Results. Syntocinon injected i.v. into anesthetized lactating rats a few minutes after the young had commenced to nurse resulted in milk let-down after a latent period of 5-8 seconds and seemed to persist 4-5 minutes. After milk flow apparently had ceased, they became quiet and sucked intermittently the remainder of the nursing period. Amount of milk thus obtained expressed as percent litter body weight is shown in Table I and is compared with data similarly obtained previously(1) with natural oxytocin. Syntocinon resulted in same magnitude of milk let-down using our 3-point method of assay, as did natural oxytocin. No milk was obtained during 30 minutes very active sucking by young of anaesthetized lactating rats injected i.v. with large doses of a relaxin preparation (Table I).

Discussion. Previous workers have found equivalent responses for natural oxytocin and Syntocinon in regard to oxytocic activity(3-5) the synthetic product having much less pressor and antidiuretic effects(3,4,6,7). The observation in the present study that Syntocinon is capable of causing milk let-down in anaesthetized lactating rats of the same magnitude as natural oxytocin indicates further the physiological similarity of the two compounds and, in addition, indicates the reliability of our method of assay in determining milk let-down. A variety of other compounds will initiate milk let-down in isolated perfused cow's udder(9,10), or will cause local alveolar contraction of mammary glands of rats, mice, guinea pigs and rabbits when applied topically(11). Recently, Shaffhausen *et al.*, (8) have suggested relaxin may also effect contraction of mammary alveoli of sheep for after obtaining all milk possible by hand milking, they were able to secure additional milk by hand milking following intraarterial injection of 500 GPU of a relaxin prepara-

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[‡] Kindly supplied by Sandoz Chemicals, Inc.

[§] Relaxin preparation W1164A of Warner-Chilcott Lab, Lot No. 53, assaying 30 GPU/mg.

TABLE I. Comparison of Milk Removal by Litters of Anaesthetized Nursling Rats Injected with Natural Oxytocin, Syntocinon and Relaxin on 14th Day Postpartum.

| Treatment | No. of rats | Avg wt (g) | | Avg % |
|--------------------------|-------------|------------|------|-----------------------------|
| | | Litters | Milk | Wt of milk Wt of litters |
| Controls* | 40 | 144.0 | 5.5 | 3.8 ± .19 |
| Nembutal† | 14 | 164.5 | .0 | .0 |
| " + 600-1200 GPU Relaxin | 10 | 133.4 | .0 | .0 |
| " + .02 USP/kg oxytocin† | 11 | 159.0 | 4.7 | 3.0 ± .82 |
| " + " " Syntocinon | 10 | 174.9 | 5.6 | 3.2 ± .21 |
| " + .05 USP/kg oxytocin† | 11 | 164.2 | 6.3 | 3.8 ± .20 |
| " + " " Syntocinon | 10 | 159.4 | 6.0 | 3.8 ± .27 |
| " + .1 USP/kg oxytocin† | 5 | 168.8 | 9.8 | 5.9 ± .45 |
| " + " " Syntocinon | 10 | 179.3 | 10.3 | 5.7 ± .46 |

* Data from Grosvenor and Turner(2).

† Data from Grosvenor and Turner(1).

tion. However, if relaxin in doses used in the present investigation was capable of contracting mammary alveoli or directly activating pituitary release of oxytocin, then nursling rats should have obtained milk. Since none was obtained, it is concluded relaxin, under the conditions of this experiment, has no milk let-down activity in the lactating rat.

Summary. 1) A synthetic oxytocin, (Syntocinon, Sandoz), injected *i.v.* into Nembutal anaesthetized lactating rats in doses of .02, .05 and .1 USP/kg a few minutes after the young had commenced to nurse resulted in milk let-down after a latent period of 5-8 seconds and seemed to persist 4-5 minutes. Amount of milk thus obtained expressed as percent litter body weight corresponded very well with data similarly obtained previously with same doses of natural oxytocin. It is concluded Syntocinon and natural oxytocin possess equivalent milk let-down activity. 2) Relaxin injected *i.v.* in dose of 600-1200

Guinea Pig Units into 10 anaesthetized lactating rats failed to initiate milk let-down.

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Erythropoietic Stimulating Activity of Urine from Anemic Human Subjects.* (23685)

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The erythropoietic stimulating activity of plasma from anemic human subjects has been repeatedly demonstrated(1-3). There are also indications that urine from anemic patients will induce erythroid marrow hyperplasia(1), elevate peripheral erythrocytic parameters(4) and increase the % incorporation of Fe^{59} into the red cells of recipient rats(3, and Mirand, personal communication).

The present studies were undertaken to determine the frequency with which erythropoietic stimulating activity appears in anemic human urine and to relate the occurrence of this activity to that of plasma.

Materials and methods. Seventeen 24-hour urine samples were collected from 6 children and 1 adult with hemoglobin levels ranging from 4 to 7 g%. Four additional samples were obtained from children with normal hemoglobin levels. All of the urines were preserved at 5°C with 5 ml of toluene added per liter. Five of the urine samples from patients with Cooley's anemia and 1 from a normal child were subjected to the acidification and boiling procedure described previously(4). The remainder of the urine samples were tested without modification. Normal mature female rats of a modified Long-Evans strain, weighing 150-200 g and maintained on a diet of Purina Chow, were used for testing the urines. Four or 5 rats were used in each assay. Each rat received 3 ml of urine injected subcutaneously once daily for 10 to 20 days.

Reticulocyte, red cell, hemoglobin and hematocrit determinations were performed prior to the injection period employing methods previously described(5). Reticulocyte counts were again made on the 6th day and red cell,

hemoglobin and hematocrit estimations were repeated on the day following the last injection. These are the values indicated in Table I. The red cell counts for each animal were taken as the mean of the 4 chamber counts drawn from 2 separate pipettes. The same pipettes were used before and after treatment for each rat. In 4 assays, bone marrow smears were prepared after the last injection following the method already reported(6). In 1 assay, blood volume determinations were performed according to a modification of the method of Wang and Hegsted(7,8).

Results. Of the 5 urine samples taken from the patients with Cooley's anemia and subjected to the acidification-boiling procedure, only the sample listed in the first row of Table I possessed erythropoietic stimulating activity. The other 4 inactive samples are not listed in the table. Similarly treated urine obtained from the normal boy was inactive.

The remaining 12 urine samples which were obtained from the anemic children and the adult with a benign thymoma, were not acidified and boiled. Eight of these evoked increases in some of the erythrocytic values (Table I). These included 2 of 4 samples obtained from Cooley's patients, 3 of 5 secured from the hypoplastic anemia subject and 1 each from the myelogenous leukemia, benign thymoma, and sickle cell anemia patients. However, only 4 of the 8 positive responses involved more than 2 erythrocytic parameters. The 4 inactive samples are not included in the table.

No erythropoietic stimulating activity was noted with a Cooley patient's urine after a transfusion had brought his hemoglobin level to 12 g%. Similarly, the urine from the myeloblastic leukemia patient in remission was inactive as was the urine obtained from the normal girl.

The 2 samples of urine collected from the

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TABLE I. Effects of Urine from Anemic Patients on Peripheral Blood of the Rat.

| Status of urine donor | | | Effects in recipient rats (mean \pm S.E.) | | | | |
|-----------------------|------------------------------------|------------------|---|-----------------------------|------------------|-----------------|----------------|
| Age (yr) | Diagnosis | Hemoglobin (g %) | | RBC (mill/mm ³) | Hemoglobin (g %) | Hemato-crit (%) | Retics (%) |
| 6 ♂ | Cooley's anemia | 5.4 | B \S | 9.3 \pm .2 | 16.0 \pm .5 | 47.2 \pm 1.0 | 2.1 \pm .2 |
| | | | A | 12.0 \pm .4† | 18.4 \pm .8* | 54.6 \pm 2.2* | 4.6 \pm .8* |
| | | 5.5 | B | 8.7 \pm .4 | 15.3 \pm .4 | 46.1 \pm 1.3 | 2.5 \pm .9 |
| | | | A | 9.7 \pm .2 | 17.7 \pm .5* | 51.7 \pm 2.6 | 7.7 \pm .8† |
| | | 4.3 | B | 9.0 \pm .2 | 14.9 \pm .2 | 46.9 \pm 1.9 | 3.9 \pm .7 |
| | | | A | 11.1 \pm .6* | 18.3 \pm .9* | 57.7 \pm 2.3* | 8.3 \pm 1.3* |
| 7 ♂ | Hypoplastic anemia | 5.5 | B | 8.5 \pm .1 | 14.7 \pm .2 | 45.6 \pm 1.2 | 3.0 \pm .4 |
| | | | A | 11.0 \pm .1† | 18.1 \pm .7† | 55.6 \pm 1.4† | 5.2 \pm .4† |
| | | 4.5 | B | 8.7 \pm .3 | 15.3 \pm .6 | 46.1 \pm 1.2 | 3.9 \pm .4 |
| | | | A | 10.3 \pm .3† | 17.1 \pm .3* | 51.0 \pm .7† | 7.7 \pm .9† |
| | | 6.0 | B | 9.0 \pm .2 | 15.9 \pm .3 | 49.8 \pm .9 | 2.9 \pm .8 |
| | | | A | 10.0 \pm .2* | 16.0 \pm .7 | 50.3 \pm .7 | 3.7 \pm .7 |
| 3 ♂ | Myeloblastic leukemia | 6.5 | B | 9.3 \pm .2 | 14.5 \pm .3 | 54.5 \pm .3 | 3.1 \pm .6 |
| | | | A | 11.6 \pm .2† | 16.5 \pm .2† | 57.0 \pm 2.1 | 6.0 \pm .4† |
| 3 ♀ | Sickle cell anemia | 6.5 | B | 9.0 \pm .2 | 14.9 \pm .2 | 49.5 \pm .5 | 2.0 \pm .4 |
| | | | A | 10.2 \pm .3* | 16.5 \pm .5* | 52.0 \pm 1.4 | 2.7 \pm .9 |
| 68 ♀ | Benign thymoma | 7.0 | B | 9.1 \pm .1 | 15.6 \pm .4 | 50.4 \pm 1.3 | 2.8 \pm .7 |
| | | | A | 10.2 \pm .1* | 16.7 \pm .3 | 51.9 \pm 1.1 | 4.6 \pm .6 |
| 3 ♂ | Myeloblastic leukemia in remission | 11.7 | B | 8.3 \pm .1 | 15.6 \pm .9 | 45.0 \pm 4.3 | 2.9 \pm 1.3 |
| | | | A | 8.6 \pm .3 | 14.9 \pm .6 | 42.0 \pm 1.0 | 3.2 \pm .9 |
| 6 ♂ | Cooley's (transfused) | 12.0 | B | 9.5 \pm .1 | 17.6 \pm .3 | 50.0 \pm 1.7 | 2.9 \pm .3 |
| | | | A | 9.0 \pm .4 | 14.8 \pm .2 | 44.0 \pm 1.0 | 3.3 \pm .4 |
| 6 ♂ | Normal | 13.5 | B | 9.2 \pm .2 | 16.6 \pm .6 | 49.6 \pm 1.2 | 2.4 \pm .3 |
| | | | A | 8.9 \pm .2 | 14.6 \pm .6† | 45.4 \pm 2.2 | 4.7 \pm .7* |
| 3 ♀ | " | 13.0 | B | 8.3 \pm .1 | 15.6 \pm .3 | 49.6 \pm .9 | 3.0 \pm .8 |
| | | | A | 8.2 \pm .2 | 14.9 \pm .7 | 46.5 \pm 1.1 | 3.2 \pm .4 |

* Mean significantly greater than pretreatment level, $P < .05$.† *Idem*‡ Mean significantly lower than pretreatment level, $P < .05$.

§ B = Before treatment; A = After treatment.

boy with hypoplastic anemia which elicited the marked increases in all 4 peripheral parameters listed in Table I (Hb 5.5 and 4.5

TABLE II. Effect of Urine from Anemic Patients on Bone Marrow of the Rat.

| Status of urine donor | | Marrow response after treatment (mean \pm S.E.) | |
|---------------------------|--------------------|---|--|
| Age | Diagnosis | Hb. (g %) | Nucleated RBC (% of total nucleated cells) |
| 6 ♂ | Cooley's anemia | 5.4 | 36.6 \pm 3.4 |
| | | 5.5 | 42.2 \pm 6.9* |
| 7 ♂ | Hypoplastic anemia | 5.5 | 45.8 \pm 1.9† |
| | | 4.5 | 44.2 \pm 4.0† |
| 3 ♀ | Sickle cell anemia | 6.5 | 27.2 \pm 7.0 |
| 6 ♂ | Normal | 13.5 | 41.0 \pm 1.5* |
| 50 untreated control rats | | | 32.4 \pm 2.4 |

* Mean significantly greater than untreated controls, $P < .05$.† *Idem*, $P < .01$.

g%) also induced erythroid marrow hyperplasia (Table II). Blood volume values, determined in the animals receiving the first of these 2 active urines, were also significantly elevated (Table III). Nucleated red cell percentages were increased in the bone marrow of rats receiving the urine of the normal boy (Table II). This was probably not a true erythropoietic stimulating effect but most likely was a secondary consequence of the fall in hemoglobin induced in the recipient rats by this urine sample (Table I).

Discussion. It is clear that some samples of urine obtained from anemic human subjects markedly augment peripheral red cell parameters in the rat. These rises, most likely, cannot be attributed to hemoconcentration since, as has been shown with 1 active sample, there was a concomitant rise in blood volume. Furthermore, it is probable that this

TABLE III. Effect of Urine from an Anemic Patient on Blood Volume of the Rat.

| Donor | Blood vol (cc/100 g body wt) |
|----------------------------------|---------------------------------|
| Hypoplastic anemia (Hb. 5.5 g %) | 10.1 \pm .3* |
| Untreated controls | 8.3 \pm .4 |

* Mean significantly greater than untreated controls, $P < .01$.

increase in the total numbers of circulating erythrocytes is the result of enhanced production since there was an associated increase in the numbers of peripheral reticulocytes and an augmentation in the % of nucleated erythrocytes in the bone marrow.

Only one-fourth of the urine samples examined, however, elicited an increase in at least 3 erythrocytic parameters. Obviously, with such a low incidence of activity in anemic human urine, more than 4 samples are required to determine whether unconcentrated urine of normal subjects is consistently inactive.

There was no remarkable difference in the occurrence of activity between urine subjected to the acidification-boiling procedure and that not so treated. The presence of activity in at least 1 sample after boiling suggests that the factor in urine is heat stable. In this respect, it is similar to the factor obtained previously from the plasma of these same patients(1). When compared to anemic human plasma(1), anemic human urine is a much less constant source of erythropoietically active material. It is probably more dilute, since more injections are required to evoke detectable responses. As a source for the isolation of the factor, urine has the advantage, however, of being readily available in large quantities relatively free from plasma proteins.

It is not certain why urine is less frequently active than plasma. Examination of the clinical records indicates no correlation between the presence of activity in urine and the general status of the patient, the severity of the anemia, or the degree of concentration (spe-

cific gravity) of the urine. It is of interest that the most frequently active urine has come from the patient with hypoplastic anemia whose plasma, at least on one occasion, has been shown to be devoid of erythropoietic activity(1).

More work is required to establish the frequency of occurrence of erythropoietic activity in normal human urine and to elucidate its variable presence in the urine of anemic subjects. In this regard, the question of normal and pathologic variations in renal threshold should be studied.

Summary. Of 17 unconcentrated urine samples taken from 7 anemic humans, approximately 25% displayed erythropoietic stimulating properties. Blood volume and bone marrow studies confirmed the erythropoietic nature of the responses obtained. Urine from 4 children with normal hemoglobin levels showed no ability to stimulate erythropoiesis. When compared to anemic human plasma, anemic human urine is a more dilute and less constant source of erythropoietically active material.

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Localized Cerebral Hypothermia.* (23686) ✓

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A number of technics have been developed for localized cooling of the brain. They have in common either total vascular isolation of the perfused brain, as described by Malméjac for instance(1), or irrigation of the brain with cooled blood through the carotid arteries with an unimpeded efferent flow into the systemic circulation of an intact animal(2-7). In several experimental applications of the latter design and in one clinical trial(8), a significant lowering of body temperature has also been noted, and brain-body circulatory mixing must be considerable. The pattern of the present study includes an intact systemic circulation and a localized extracorporeal cerebral circulation whose afferent and efferent channels are controlled through the carotid arteries and jugular veins by a dual channel, independently variable, torque conversion Sigmamotor pump.

Methods. The perfusion unit consisted of a 500 cc bubble oxygenator into which blood and 100% oxygen were pumped, a Monel metal helix heat-exchanger immersed in ice water and stainless steel filters, included in the circuit to remove fibrin clots. Bead thermistors were used to record temperature gradients in the extradural space, the esophagus and rectum. An electromagnetic Rotameter was used to record perfusion flow and perfusion pressure was measured by a Sanborn electromanometer. Paired silver electrodes were placed in the frontal and occipital extradural areas and standard limb EKG leads were used. The systemic arterial pressure was measured by a Statham strain gauge. These observations were recorded on a 6 channel Offner Polygraph and a 4 channel Sanborn Polyviso. The systemic venous pressure was measured on a standard water manometer. Hematocrit, clotting-time and arterio-venous blood oxygen samples were obtained from appropriate areas of both circu-

lations. Mongrel dogs were used and 43 studies have been carried out in the continuing development of this technic. Each animal was anesthetized with nembutal, 20 mg per kg, and placed on a Palmer respirator giving 400 cc of 100% oxygen per minute through an endotracheal tube. After operative preparation of the recording sites, both carotid arteries and both external jugular veins were prepared for cannulation. The extracorporeal circulation unit was primed with 1500 cc of donor blood secured 24 hours previously by exsanguination. Both jugular veins were cannulated and connected with a venous reservoir through gravitational flow. The carotid arteries were cannulated in sequence and the perfusion started. Each animal received 25 mg of heparin. At the end of the perfusion period, ranging from 40 minutes to 3 hours and 55 minutes, the jugular veins were ligated, the carotid arteries repaired and rewarming occurred through the systemic circulation. The extradural electrodes were left in place. EEG recordings were taken at survival periods of 5 to 33 days, the animals sacrificed and their brains studied by standard neuropathological methods.

Results. The localized brain cooling produced by this technic possessed the properties of rapid induction and ready reversibility and results obtained were reproducible in all parameters of study within the limit of technical errors. Perfusion flow, or perfusion pressure, was the single important factor in terms of survival. Flow rates above 18 to 20 cc per minute per kg resulted in high systemic venous pressures, cardiac failure and autopsy evidence of cerebral edema. In the normothermic animal, perfusion flows of 6 to 7 cc per minute per kg allowed survival. In the hypothermic brain, flow values of 10 cc per minute per kg permitted the perfusion pressure to approximate the value of the systemic carotid pressure and these animals did well.

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With perfusion flows of 6 to 10 cc per min. per kg, brain temperature dropped an average of 10 degrees in 30 minutes and reached levels of 21° to 20°C after 60 minutes of perfusion. There was a linear relationship between brain cooling and the systemic blood pressure and at brain temperatures of 15° to 20°C, the mean systemic arterial pressure might range between 30 and 50 mm of mercury. The changes in cortical electroactivity noted in this form of localized brain cooling were identical to those described in generalized hypothermia(9) or in states of hibernation(10). Slow wave activity became apparent after cooling the brain to 32°C. There was a concomitant fall in amplitude as the temperature was reduced and the recordings became flat at 21° to 20°C. The cortex remained electrically silent below this temperature and when the gradient was reversed, small bursts of low amplitude activity reappeared at 21°C. At 30°C, fast activity dominated the pattern and the record became normal again at the pre-cooling temperature level. No abnormal changes were noted in surviving animals.

The principal finding in the electrocardiogram was a relative bradycardia that developed with progressive cooling. The average cardiac rate at the beginning of perfusion was 156 and was reduced to 88 when electrocortical activity ceased. Associated with the decrease in rate, the QT interval was prolonged from a pre-cooling average of .23 second to an average of .43 second when the brain temperature reached 21°C. There were no instances of ventricular fibrillations or other cardiac arrhythmias. The rectal temperature fell from an average of 38°C to one of 32°C and was dependent upon the length of perfusion. Low body temperatures usually occurred at the end of perfusion periods when rewarming of the brain was carried out by the animal's systemic circulation.

The arterio-venous oxygen difference in the perfusion blood of the cerebral circulation fell in a linear fashion with decreasing brain temperatures and reached 0.0 at a brain temperature of 21° to 20°C. There was little change in AVO difference in the systemic circulation unless the animal's body temperature

at the stage of rewarming fell below 34°C. Brain-body circulatory mixing could be demonstrated by the addition of 30 μ c of radioiodine labelled serum albumen in the blood volume to be circulated through the brain. By conventional radioanalysis methods, the percentage of circulatory mixing could be determined and, in 3 animals, this measured between 15 and 20%. In 6 animals, the blood perfusate was replaced by Ringer's solution at a brain temperature of 20°C, and venous return hematocrits from the brain measured at 5-minute intervals at a constant perfusion pressure. These fell from 14% during the first 5 minutes of perfusion to 5% at the end of 30 minutes, again suggesting a collateral circulation approximating 15%. At these flow levels, no evidence of air embolism as a result of blood oxygenation could be demonstrated.

Conclusion. Localized cerebral hypothermia has been produced in the dog by an extracorporeal circulation utilizing the carotid arteries and external jugular veins. During the acute stage of perfusion, various parameters of study have been recorded and in general, these results are in accord with those noted in states of generalized hypothermia. Survival is predicated upon low perfusion flows and development of perfusion pressure closely aligned with the systemic arterial pressure. Such long-term survivals allow the further investigation of the effect of various drugs and chemical substances upon the normothermic and hypothermic brain whose circulation is isolated in a measurable manner from the systemic circulation.

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Modification of Sex Differentiation by Steroid Hormones in a Tree Frog (*Pseudacris nigrita triseriata* Wied).^{*} (23687)

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Amphibians have been used most successfully in experiments on hormonal modification of the processes of sex differentiation. While results obtained with different taxonomic orders and families first seemed irregular or even contradictory, accumulation of a considerable body of data is now suggesting a correlation between genic sex type and mode of response(1). In view of the possible bearing of this relationship on the theory of gene action, verification by further extension of the experiments to still untested groups is desirable. The present report on a tree frog bears on a family (hylidae) which, according to Yosida (2) and the senior author's own observations, seems chromosomally related to the family of the common frogs (ranidae).

Material and methods. Normally deposited eggs were collected near Iowa City and kept in finger bowls until hatching of the tiny larvae. On the day when feeding started, groups were separated according to the planned hormone treatments. The experimental lots were placed and reared in steroid solutions of the concentrations indicated in Table I. Mortality was moderate, too low to affect the outcome of the tests.

Results. Testosterone propionate is completely masculinizing the genetically female half of the treated groups. The used concentrations of 50 and 500 $\mu\text{g/l}$ obviously are not limit values, but they are sufficiently apart to

permit the conclusion that all effective dosages are masculinizing. The study of normal sex differentiation of the same species by Deal (3) revealed that all male gonads are invaded by numerous pigment cells during and immediately following initial sex differentiation (larvae 15 to 18 mm). They move along with ingrowing mesonephric blastema cells. Ovaries never are pigmented. In the experimental transformation of genetic females into males, in the present experiments, the testes became as darkly pigmented as those of genetic males. In contrast to the persistently masculinizing action of testosterone, the effect of estradiol (and estrone) treatments depends on the dosage level; it is feminization at 50 $\mu\text{g/l}$, but an almost normal sex ratio at the higher level (Table I).

Discussion. The described experiments with treefrogs give results that are essentially identical with those obtained in more extensive tests with *Rana sylvatica*(4). The slowly but steadily accumulating data on reactions of amphibians belonging to various taxonomic orders and families permit now to distinguish

TABLE I. Effect of Continuous Steroid Hormone Administrations on Sex Differentiation in *Pseudacris*. Treatments started after closure of gill sacs (Stage 25). All animals preserved and sexed at end of metamorphosis (Stage 33).

| Hormone | | Females | Inter- sexes | Males |
|--------------|--------------------|---------|-----------------|-------|
| Testosterone | 50 $\mu\text{g/l}$ | | | 27 |
| " | 500 " | | | 209 |
| Estradiol | 50 " | 16 | | |
| Estradiol | 100 " | | | |
| + Estrone | 200 " | 104 | 1 | 96 |
| Controls | | 37 | | 33 |

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2 reaction types (for detailed bibliography consult ref. 1). In the first type, androgens produce complete and durable masculinization of genetically female embryos; gynogens are feminizing at low effective dosages—apparently effecting only an unstable sex inversion of genic males—but at high dosages they are masculinizing genic females. To this type belonged, so far, only the ranids; they are now joined by the hylids. In the second reaction type, represented by several families of primitive anurans (discoglossids, xenopins) and all urodeles, androgens produce either no major effects or, in some instances, they feminize genic males; gynogens however are feminizing the larvae of male genic constitution.

Experiments with the common toad indicate that in general bufonids follow the second reaction type. However, feminization by gynogens is unstable(5), so that in this regard they seem related to the first type. Their intermediary behavior is of some interest in view of the taxonomic position of the bufonids between the higher and the primitive families of the anurans.

Breeding experiments with various sex races and with sex reversed animals so far have shown that in frogs the males are heterozygous for sex determining genes, while in

toads, all lower anurans, and salamanders the females are the heterozygous sex. The number of fully investigated species is still small, but all cases conform to the rule that the first reaction type (to steroids) is exhibited by the species with male heterozygosity and the second by those with female heterozygosity. The remarkable parallelism points to some characteristic difference in the mechanisms of gene action in the two types.

Summary. Testosterone masculinizes the course of gonad differentiation in genetically female larvae of treefrogs. Gynogenic steroids are only partially feminizing the gonadal development of genetic males. These results establish for hylids and ranids a common reaction type to steroid treatments, which differs characteristically from that of all other amphibian families which, so far, were submitted to tests.

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Copulatory Reflex by Progesterone and Desoxycorticosterone Acetate.* (23688)

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Using the persistent estrous rat as a test animal, it has been shown that progesterone (1) and desoxycorticosterone acetate (DOCA)(2) are equally effective in restoring estrous cycles. Other earlier reports(3,4,5) had established that DOCA is 1/10 to 1/15 as potent as progesterone in a variety of tests. Most recently using the elicitation of the copulatory reflex in guinea pigs, DOCA was

found to be $\frac{1}{4}$ as potent as progesterone(6). Studies in progress in our laboratory for several years have indicated that the relative activity of these 2 substances is dependent upon the absolute amount used in the test, and have lead to this report.

Methods. Young, adult, female guinea pigs were ovariectomized and allowed to recover for 30 days. The completeness of the surgery was attested to by examination of the extirpated ovaries, lack of estrous behavior in untreated animals, and examination of the

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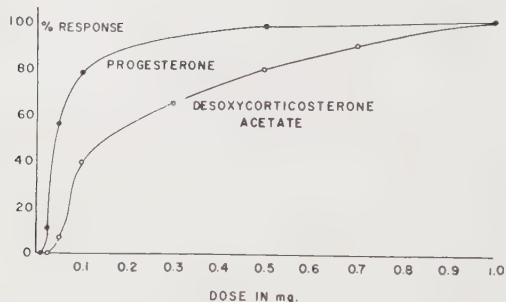


FIG. 1. Curvilinear relationship of dose-response curves of progesterone and desoxycorticosterone acetate.

operative site at time of sacrifice. The testing procedure used was essentially the same as the standard reported method(7), except: a single priming dose of 5 μ g of estrone in 0.05 ml corn oil was given 24 hours before the test material, and the test material was dissolved in 0.10 ml of oil. All injections were subcutaneous. Each animal was "rested" 30 days between tests, and none was used for more than 12 tests. Hourly tests were carried out for a minimum of 10 hours, or until responding animals failed to respond for 2 hours in succession.

Results. The points with which the curves in Fig. 1 were drawn represent 20-30 animals each. In every case, doses of the 2 steroids larger than 1 mg resulted in all animals giving strong, positive copulatory responses. A latent period between the injection of the test material and the first copulatory response characterized all reacting animals. With progesterone this latent period decreased from an average of 4 hours to 2.7 hours over a dosage range of 0.025 mg to 1.0 mg. Similarly with DOCA, the length of this period decreased from 5 hours to 2.9 hours over a dose range of 0.05 mg to 5.0 mg. In contrast to this similarity, the amount of progesterone injected had little effect on the duration of responsiveness (6.2 hours at 0.025 mg to 7.0 hours at 1.0 mg), while the amount of DOCA significantly affected the duration of responsiveness (2.0 hours at 0.05 mg to 8.1 hours at 1.0 mg).

Our results with progesterone agree well with those of others(6), 56% of 25 animals responding in our series on 0.05 mg as com-

pared with 60% of 30 animals in the reported series. Our results emphasize the necessity of comparing dose-response curves over a range of doses, since DOCA is here shown to have 13-100% the effectiveness of progesterone depending upon the absolute level of comparison.

Examination of the curves suggests another point for consideration. The progesterone curve is typically a sigmoid, biologic dose-response curve, but the DOCA curve is approximately linear above the 0.1 mg dose level. This difference may mean that an additional reaction is interposed between the absorption of the injected DOCA, and the action on the end organ. It is possible, perhaps, that the DOCA must be converted to progesterone metabolically before the reflex can be elicited. This hypothesis is supported by the greater latent period of DOCA, and by the fact that the duration of response increases directly as the dose.

Pregnenolone acetate was tested at only the 50 mg level, with negative results in 8 guinea pigs.

Summary. Using the elicitation of the copulatory reflex as a criterion, progesterone and desoxycorticosterone acetate were compared. The latter was found to be 13-100% as effective as progesterone over a range of 0.025-1.0 mg. With both substances, the latent period varied inversely as the dose, but duration of responsiveness varied directly with dosage only in the case of desoxycorticosterone acetate.

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Coagulation During Hypothermia in Man.* (23689)

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The possibility of disturbances in coagulation during hypothermia is well recognized. It has been demonstrated that total body cooling in dogs is associated with marked disturbances in *in vitro* clotting tests(1,2,3) and frequently with frank hemorrhage(2). On the other hand, hemorrhage has not been a common problem in man during or after surgery performed at hypothermic temperatures. In the present report coagulation studies are presented on 10 patients before and after slow cooling and during surgery at lowered body temperature. Clotting time, platelet count, thromboplastin generation, prothrombin consumption, prothrombin time (Quick), and prothrombin, accelerator globulin, proconvertin and fibrinogen concentrations were determined. Although the clotting times of various coagulation tests were prolonged with blood or plasma incubated at the lowered body temperatures, the concentration of coagulation factors remained essentially normal during uncomplicated hypothermia (Temperature 29.1°C-31.0°C).

Material and methods. Ten patients were studied, 5 women and 5 men. Their ages ranged between 18 and 70 years. The diagnoses were cerebral vascular aneurysm (4 patients), meningioma (3 patients), cerebellar hemangioblastoma (1 patient), and thoracic aortic aneurysm (2 patients). Anesthesia was induced with intravenous thiopental sodium and nitrous oxide. Intubation was accomplished with the assistance of intravenous succinyl-choline 40-60 mg. The patients were cooled slowly (3-4 hours) by means of surface ice packs and rendered apneic with intravenous d-tubocurarine to prevent shivering. Hyperventilation was achieved by controlled respiration with the Jefferson Venti-

lator. Intravenous fluids during the period of cooling were limited to a slow infusion of 200-400 cc normal saline or 5% dextrose in water. An intravenous infusion of Arfonad (trime-thaphan) 0.1% was used in 5 patients to control excessive blood pressure elevation. *Blood samples* were drawn: (1) after induction of anesthesia but before cooling; (2) after 3-4 hours of cooling, shortly before or after the beginning of surgery, and before the transfusion of blood; esophageal temperature at this time varied between 29.1°C-31.0°C; (3) near the end of surgery, 3-5 hours after second sample, temperature 27.3°-30.8°; 500-4000 cc of fresh blood collected by passage across the Fenwal cation exchange resin was administered between samples No. 2 and 3. Blood was obtained using untreated syringes and needles. When plasma was required, 9 volumes of blood were mixed with 1 volume of anticoagulant.† The cells were sedimented at 2000 rpm‡ for 20 min. in ordinary glass tubes. Thromboplastin generation was assayed by the method of Biggs and Douglas(4), as modified by Alexander and Goldstein. Asolectin was used as a substitute for platelets. Prothrombin consumption was determined by the method of Alexander(5). Platelets were counted by the direct Rees and Ecker method (6). Prothrombic activity was studied by the Quick one-stage method using Simplastin§ as a source of calcium and tissue thromboplastin(7). Accelerator globulin was determined by the method of Alexander(8). Proconvertin and prothrombin concentrations were determined by a modification of Owren's method(5). Fibrinogen was determined quantitatively by the method of Cullen and Van

† 0.1M sodium oxalate was used for thromboplastin generation test, and 3.8% sodium citrate for prothrombin, proconvertin and Ac-globulin tests.

‡ International Centrifuge, Size 1, Type SX, International Equipment Co., Boston.

§ Available from Warner-Chilcott Lab.

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Slyke(9). Fibrinolytic activity was estimated by inspection of the whole blood clot after 1, 2, 4, 12 and 24 hours of incubation at 37.5°C. Coagulation tests were carried out in a water bath at 37.5°C and at the temperature of the patient. Only data obtained at 37.5°C are included in the table.

Results are presented in Table I. In the absence of surgery or blood transfusion, hypothermia (29.1°-31.0°) was not associated with significant disturbances in any of the specific clotting factors measured. During hypothermia, surgery and transfusions, a variety of moderate disturbances in factors of the 1st and 2nd stages of coagulation was observed. With the exception of depression of prothrombin consumption, these disturbances were similar to those observed by us during surgery and transfusions in patients at normal body temperature. The depression of prothrombin consumption, marked in 3 and moderate in 3 other patients, could not be explained by the degree of thrombocytopenia, or by the alteration in thromboplastin generation encountered in 2 patients. However, a qualitative change in platelets was not ruled out, and plasma factors involved in thromboplastin formation were not measured individually.

Prolongation of the clotting times for all coagulation tests except whole blood clotting times was consistently observed when performed at the hypothermic temperatures rather than at 37.5°C.

Postoperative clotting studies were performed in 2 patients: 3 hours, 1, 3, and 7 days following surgery in one; 1, 2, and 7 days in the other. Moderate depression of platelets and Ac-globulin, present at the end of surgery in one of the 2, persisted for the first and second postoperative days, but returned to normal or somewhat above normal levels at the end of one week. Both patients demonstrated moderate increases in most clotting factors by the 7th day, and one showed a moderate thrombocytosis (264,000 preop. to 416,000 on 7th day).

Discussion. The normal concentration of coagulation factors during uncomplicated hypothermia observed by us in man clearly differs from results of similar studies in the experimental animal. Moderate to marked

depression in platelets(1,2) and in accelerator globulin, proconvertin, prothrombin, and fibrinogen(3) have been described in the hypothermic dog. Whether the difference in findings is due to the lower temperature induced in most of the animal experiments (20-25° compared to 27-31°C) or to a species difference is not known. That the degree of hypothermia may be a significant factor is suggested by animal experiments in which the degree of thrombocytopenia was directly related to the degree of hypothermia(1). Marked thrombocytopenia usually occurred only below 25°C and was then associated with marked elevation in clotting times and depression of prothrombin consumption. However, in dogs, even at the temperatures used for hypothermia in man, more constant depression in platelets is observed and hemorrhage has been encountered; thus it seems likely that a species difference may exist, since hemorrhage is an unusual complication of hypothermia in human beings. There has been no evidence of unusual bleeding during surgery, and no postoperative hemorrhage, in any of 28 patients who have been subjected to hypothermia in this hospital. Occasional postoperative hemorrhage has been reported by Swan(10), who concluded that the "hypotension accompanying hypothermia" may have prevented bleeding from cut vessels which later bled when pressure returned to normal. In our experience, based on direct pressure tracings from an inlying arterial needle, the blood pressure often rises, but rarely falls during hypothermia. It is perhaps more likely that the occasional occurrence of posthypothermia bleeding is secondary to reflex vasodilation of subcutaneous vessels during the rewarming period.

It should be mentioned that an intravenous infusion of Arfonad (trimethaphan) was used in 5 of the 10 patients to control marked elevations in systolic blood pressure. Arfonad has been reported by Helmsworth(11) to afford partial protection against the thrombocytopenia of hypothermia in dogs. No correlation could be found in the present studies between Arfonad administration and the degree of thrombocytopenia (Table I).

Although we have been unable to find evi-

TABLE I. Coagulation Factors during Hypothermia.

| Case No. | Temp. at sampling (°C) | | | Clotting time (min.) | | | Platelets (1000/mm ³) | | | Prothrombin consumption (%) | | | Thromboplastin generation [§] | | | Quick prothrombin activity (%) | | | Owren prothrombin conc. (%) | | | Accelerator globulin (%) | | | Proconvertin (%) | | | Fibrinogen (g %) [†] | | |
|----------|------------------------|------|------|----------------------|----|----|-----------------------------------|------|------|-----------------------------|----|----|--|---|---|--------------------------------|-----|----|-----------------------------|-----|-----|--------------------------|-----|-----|------------------|-----|-----|-------------------------------|----|----|
| | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C | A | B | C |
| | 37.5 | 29.5 | 28.8 | 13 | 13 | 11 | 252 | 452 | 212* | 86 | 99 | 99 | | | | 78 | 98 | 70 | 78 | 78 | 70 | | | | 100 | 100 | 74 | 52 | 52 | 40 |
| 1 | 36 | 31.5 | 27 | 15 | 9 | 14 | 328 | 336 | 132 | 92 | 81 | 56 | | | | 100 | 88 | 85 | 90 | 83 | 95 | 100 | 81 | 60 | 100 | 82 | 92 | 46 | 41 | |
| 2 | 37.5 | 29.8 | 28.4 | 12 | 12 | 12 | 226 | 180* | 82 | 96 | 73 | 54 | N | N | | 97 | 100 | 88 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 86 | 43 | 41 | 28 |
| 3 | 37.5 | 31 | 28 | 11 | 6 | 10 | 282 | 316 | 188 | 94 | 88 | 56 | N | N | A | | | | 100 | 100 | 80 | 100 | 100 | 84 | 100 | 100 | 80 | 43 | 41 | |
| 4 | 36 | 29.1 | 30.6 | 10 | 12 | 11 | 124 | 102 | 120† | 90 | 91 | 68 | | | | 68 | | 58 | 100 | 100 | 100 | 30 | 28 | 13 | 62 | 33 | 36 | 35 | 20 | |
| 5 | 36 | 30.2 | | 11 | 13 | | 178 | | 119 | 83 | 70 | | N | N | | 76 | 65 | 29 | 98 | | 88 | 96 | 84 | 76 | 72 | | | | | |
| 6 | 36 | 31 | 27.3 | 12 | 15 | 19 | 173 | 127* | 119* | 95 | 91 | 74 | N | N | | 90 | | 70 | 100 | 100 | 100 | 100 | 94 | 74 | 100 | 86 | 80 | 29 | 27 | 32 |
| 7 | 37.5 | 28.2 | | 15 | 12 | | 269 | | 123† | | | | N | A | | | | | | | 96 | 92 | 64 | 98 | 79 | | 47 | 33 | | |
| 8 | 37 | 30.9 | 30.8 | 12 | 9 | 14 | 264 | 324 | 258 | 98 | 94 | 94 | N | N | | 100 | 100 | 96 | 100 | 86 | 92 | 100 | 100 | 100 | 86 | 100 | 100 | 53 | 46 | 42 |
| 9 | 37 | 30.5 | 28.7 | 14 | 11 | 11 | 220 | 322 | 182 | 99 | 93 | 91 | N | N | | 100 | 51 | 83 | 100 | 94 | 90 | 100 | 98 | 75 | 100 | 98 | 100 | 86 | 72 | 50 |
| 10 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

A = before cooling; B = hypothermia, no transfusion; C = hypothermia, surgery, and transfusion.

* Patient receiving Arfonad (trimethaphan) 0.1% infusion at time of sample.

† No fibrinolysis observed in any patient.

|| Craniotomy.

‡ Patient received Arfonad earlier, but not at time of sample.

¶ Aortic graft.

dence that the concentrations of clotting factors fall during hypothermia in man, it is clear that the "activity" of all factors is depressed at these lower temperatures. It is, perhaps, surprising that frank bleeding does not occur as a result; on the other hand, it may be fortunate that a temporary slowing of coagulation does occur to lessen the possible danger of thrombosis occasioned by the slowing of circulation which accompanies hypothermia (12).

Marked shortening of whole blood clotting times in patients 2 and 4 (Table I) suggested the possibility of hypercoagulability or perhaps intravascular coagulation, but there were no other coagulation changes to support such a hypothesis. Similar shortening of whole blood clotting time has been observed by us in approximately the same frequency in normothermic patients undergoing anesthesia and surgery.

The possibility of thrombosis is important to consider and "intravascular clotting" has been implicated by Ellis as the explanation for the fall in all coagulation factors he observed in dogs(3). However, recently it has been demonstrated that the drop in platelets in dogs is due to a sequestration of platelets and not to their consumption or destruction (13). In our series of patients, 4 suffered postoperative thrombosis of cerebral vessels. Operative trauma during craniotomy was thought to be the causative factor, but a slowed cerebral flow may have contributed. The rôle of increased concentrations of clotting factors, observed in 2 patients postoperatively, must also be considered. However, there is no evidence at present that the "hypercoagulability" after hypothermia is different from that following any operative procedure.

Summary. Clotting times, platelets, thromboplastin generation, prothrombin consumption, prothrombin time, and concentrations of prothrombin, accelerator globulin, proconvertin, and fibrinogen were measured in 10 patients after the slow induction of hypothermia and later during surgery and transfusion. In contrast to previous studies in the dog, uncomplicated hypothermia in man was not as-

sociated with a fall in concentration of clotting factors. Moderate disturbances in coagulation with surgery and transfusion during hypothermia were in most regards similar to those observed during surgery and transfusion at normal body temperature. The one significant difference was a decrease in prothrombin consumption.

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Potentiating Effects of Prenatal X-Irradiation on Dental Caries in the Rat.* (23690)

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It is well established that x-irradiation during the prenatal period will result in abnormalities or death of the young depending on the time of gestation and the dosage of x-irradiation administered. The pertinent literature in this field has been extensively reviewed by Russell(1). These studies, however, have dealt primarily with the effects of x-irradiation on the young as judged by their morphological appearance or viability at the time of parturition or the period immediately following. Comparatively few studies have been conducted to determine the effects of x-irradiation during prenatal life on the subsequent post-weaning response of the young. Growth retardation, brain damage and damage of the retina, cord and ganglia, sterility, skin defects and cataracts, and impaired maze learning performance(1-3) have been observed in the

surviving young of mother rats administered sublethal doses of x-irradiation during various periods of pregnancy. In the present communication data are presented on the effects of x-irradiation during the formative period of tooth development (18th, 14th or 10th day of pregnancy) on susceptibility to dental caries in the rat.

Procedure. Forty female rats of the Long-Evans strain which had been raised from weaning on a natural food stock ration† were selected at 3 to 4 months of age and an average body weight of 188 g. Animals were divided at random into 4 equal groups of 10 rats each and were mated to males of proven fertility. Pregnancy was dated by the finding of sperm in the vaginal smear.‡ Animals in Group I served as non-irradiated controls; those in Groups II, III and IV received a single exposure of 150 r x-irradiation on the 18th, 14th or 10th day of pregnancy respec-

* This investigation was supported in part by Medical Research Contract No. DA-49-007-MD-768 with the Surgeon General's Office, Department of Army, Washington, D. C.

† Rockland Rat Diet, Arcady Farms Milling Co., Chicago, Ill.

TABLE I. Effects of a Single Dose of 150 r X-irradiation during Various Periods of Prenatal Life on the Reproductive and Lactation Performance of Rats.

| | Non-irrad. | Irradiated on following day of pregnancy: | | |
|---|------------|---|------------|------------|
| | | 18th day | 14th day | 10th day |
| No. of rats bred | 10 | 10 | 10 | 10 |
| No. of litters cast | 9 | 9 | 10 | 10 |
| Avg litter size*† | 7.8 ± 1.1 | 7.7 ± .7 | 9.3 ± .5 | 7.6 ± .9 |
| % young born dead | 1.8 | 0 | 0 | 0 |
| Avg birth wt of young, g* | 6.1 ± .3 | 5.6 ± .2 | 5.5 ± .2 | 5.4 ± .2 |
| % mortality of young during lactation period | 7.0 | 9.8 | 9.6 | 39.5 |
| % incidence of microphthalmia and anophthalmia in young | 0 | 0 | 0 | 40.0 |
| Avg weaning wt of young, g* | 45.0 ± 1.4 | 40.3 ± 1.6 | 34.9 ± 2.7 | 44.4 ± 1.6 |

* Including stand. error of mean.

† Including young born dead.

tively. The radiation factors were as follows: GE Model Maximar 250; 250 kv; 15 ma; 0.5 mm Cu and 1 mm Al filters plus a Cu parabolic filter;§ HVL, 2.15 mm Cu; target distance to top of box, 78 cm; and dose rate, 17.92 r per minute (measured in air). The animals to be irradiated were placed in a wooden box divided into 14 compartments 7 cm wide, 16 cm long and 10 cm deep. The partitions and top were made of 1/8" cellulose acetate sheeting; and the top, one side and bottom of each compartment were perforated with holes for purposes of ventilation. The container was rotated slowly on an electrically-driven turntable to insure equivalent irradiation. Throughout pregnancy and lactation rats were continued on the same natural food ration on which they had been raised since weaning.† Food and water were provided *ad libitum*. No significant difference was observed between any of the groups in respect to the number of litters cast, the average number of young born or the average weight of the young at birth. The mortality of the young during the lactation period was

greater for the offspring of Group IV (rats irradiated on the tenth day of pregnancy) than for those of other groups. The average weight of surviving young of Group IV, however, when weaned at 21 days of age, did not differ significantly from the weaning weight of the young of other groups.‖ In agreement with earlier findings(1,4), 40% of the young of Group IV exhibited anophthalmia or microphthalmia in contrast to a 0% incidence of these deformities among the offspring of the other groups. Findings are summarized in Table I.

Twelve male and 12 female rats (consisting of at least 1 male and 1 female from each litter) were selected at weaning from the young of each of the above groups. Animals were placed in metal cages with raised screen bottoms (3 animals per cage) and were fed *ad libitum* a cariogenic skim milk-containing ration similar to diet 635 of McClure and Folk (5). This ration consisted of roller process skim milk powder, 35%; glucose, 18%; corn starch, 45%; and dehydrated liver, 2%. To

‡ The start of pregnancy was timed from the time that sperm were actually observed in the vaginal smear. Inasmuch as mating may have occurred at any time from 5 P.M. of the previous day to 8 A.M. of the day that sperm were found, fertilization may actually have occurred as much as 15 hours before the time that sperm were observed. The first day of pregnancy covered the first 24 hours following finding of sperm. It is estimated that actual time of x-irradiation was approximately 17½, 13½ and 9½ days post fertilization.

§ A non-uniform filter which produces a flat isodose surface of X-ray intensity constructed by the method of Greenfield and Hand(9). We are indebted to Dr. M. Greenfield and Miss Katherine Hand of the Atomic Energy Project, University of California at Los Angeles, for construction of the parabolic filter. The center of filter was 1.7 mm Cu, the edge, 0.5 mm Cu thick.

‖ The normality in weaning weight of this group may be deceptive since such a high proportion died and the survivors thus had a better chance for weight gain than did young of other groups.

TABLE II. Effects of Prenatal X-irradiation on Incidence and Severity of Dental Caries in Rats Subsequently Fed a Cariogenic Skim Milk-Containing Ration.

| Exp. group | Non-irrad. | Offspring of rats irradiated on following day of pregnancy: | | |
|--|------------|---|----------|----------|
| | | 18th day | 14th day | 10th day |
| No. of rats | 24 | 24 | 24 | 24 |
| Initial wt, g | 41.2 | 41.4 | 41.4 | 41.2 |
| Final wt, g* | 281.2 | 234.5 | 216.4 | 238.2 |
| Carious incidence (%) | | | | |
| Carious rats | 58.3 | 45.8 | 62.5 | 70.8 |
| Occlusal carious rats† | 29.2 | 20.8 | 58.3 | 54.2 |
| Surface " " | 58.3 | 37.5 | 62.5 | 58.3 |
| Caries distribution and severity (No./rat) | | | | |
| Occlusal carious areas | .6 | .3 | 1.9 | 3.3 |
| Lingual " " | .0 | .0 | .0 | 1.5 |
| Buccal " " | 1.3 | .5 | 2.2 | 4.0 |
| Score/rat‡ | 1.8 | .8 | 9.3 | 23.1 |
| Score/carious rat‡ | 3.1 | 1.8 | 15.4 | 32.0 |

* After 90 days feeding, animals were sacrificed and evaluated for caries.

† Data were based on No. of rats with occlusal caries irrespective of whether or not surface caries were also present.

‡ The caries lesions were recorded and scored as follows: occlusal, lingual and buccal surfaces were each divided into 3 areas in the case of the 1st molar and 2 areas in the case of the 2nd and 3rd molars and each carious defect was scored one, 2 or 3, according to the extent of the carious destruction. The maximum possible caries score for a 1st molar tooth was therefore $3 \times 3 \times 3$ or 27, whereas the maximum possible caries score for a 2nd or 3rd molar was $3 \times 2 \times 3$ or 18. The maximum possible caries score/rat based on evaluations of the lower teeth only (2 each of the 1st, 2nd and 3rd molars; incisors were not included) was therefore 126. "Score/rat" was determined by dividing the total caries score by the No. of animals in the group. "Score/carious rat" was obtained by dividing the total caries score by the No. of carious rats.

each kg of the above were added 5000 U.S.P. units of Vit. A, 500 U.S.P. units of Vit. D₂ and 100 mg alpha-tocopherol acetate. The vitamins were added in place of an equal amount of corn starch. Previous findings by McClure and co-workers(5,6) indicate that rats fed a ration similar to the above (diet 635) develop a high incidence of surface type caries similar in a number of respects to the type of dental caries found in man(7,8). In addition 12 male and 12 female rats similar to the above were selected at weaning from the young of each group and were continued on the same ration that had been fed during

pregnancy and lactation.† After 90 days of *ad libitum* feeding, all rats were sacrificed; the heads were autoclaved, cleaned of adhering soft tissues; and the lower teeth were evaluated for caries according to the procedure of McClure and Folk(5).

Results. Findings indicate that x-irradiation during certain periods of prenatal development significantly increased the severity of dental caries in rats subsequently fed a cariogenic skim milk-containing ration (Table II). The effects of x-irradiation were most marked on the offspring of rats irradiated on the 10th day of pregnancy. 70.8% of the rats in the latter group developed caries when fed the skim milk-containing ration with an average severity score of 32.0 per carious rat in contrast to a 58.3% incidence of caries and an average severity score of 3.1 per carious rat for the offspring of non-irradiated rats fed a similar diet.¶

It would appear from these findings that x-irradiation enhanced the severity of the carious lesion but had little if any effect on its incidence. The offspring of rats irradiated on the 14th day of pregnancy also exhibited a significant increase in severity of caries when fed the skim milk-containing ration (average severity score, 15.4 per carious rat). In contrast to the above results, no increase in the severity of dental caries occurred in rats whose mothers were irradiated on the 18th day of pregnancy. Whereas x-irradiation during certain periods of prenatal development significantly increased the severity of dental caries in rats fed a cariogenic skim milk-containing ration, it had no such effect on rats maintained on the stock ration.† The incidence of caries in rats fed the latter diet did not exceed 10% in any of the groups, with such lesions as did occur being minimal in severity.

Summary. X-irradiation during certain periods of prenatal development significantly increased the severity of dental caries in rats subsequently fed a cariogenic skim milk-containing ration. The offspring of rats admin-

¶ Distribution of caries lesion and increase in caries severity occurred with relative uniformity in all mandibular molars.

istered a single dose of 150 r x-irradiation on the 10th or 14th day of pregnancy showed a significant increase in severity of caries over that of the offspring of non-irradiated rats. Severity of caries was not increased in the offspring of rats administered a similar dose of x-irradiation on the 18th day of pregnancy.

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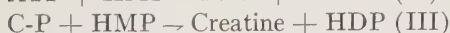
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Creatine-Phosphate Utilization by Muscle Extracts of Rabbits on Vit. E-Deficient Diets.* (23691)

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(Introduced by Stewart Wolf)

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The main pathway for creatine metabolism is presumed to be reaction I which is linked to glycolysis by reaction II. The overall reaction III was studied by following stabilization of phosphate from creatine-phosphate.



Since phosphate of creatine-phosphate also may be transferred to HMP with production of creatinine as measured by the Jaffe reaction(1), creatinine production was determined. It was necessary to ascertain that the effects observed in utilization of creatine-phosphate by muscle from Vit. E-deficient

animals were specific for the system by which C-P is used. To observe whether generalized and similar changes occurred in other metabolic pathways in muscle, activity of another soluble enzyme, phosphoglucomutase, and rate of glycolysis by muscle strips were also investigated.

Materials and methods. Animals and diets. New Zealand male rabbits weighing 500-600 g were used. One group of 26 animals was fed Mason and Harris diet(2), and to half of them a supplement of cod liver oil (4 ml/week) was given while the other half received 75 mg of α -tocopheryl acetate (Distillation Products Industries)/week. A second group of 60 rabbits received the diet shown in Table I. Creatinuria (Cr/Cn values varied from 1 to about 5) occurred after 18 days on Mason-Harris diet in animals not receiving tocopherol. Neither of these diets produced a severe dystrophy, but instead a relatively mild effect on muscle in terms of weakness and wasting, similar to that reported with diets R-14 and R-20, by Hove and Copeland(3). The animals on Diet II survived for 100 days or more and, although they became weak,

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† The following abbreviations will be used: C-P creatine-phosphate, ADP adenosine diphosphate, ATP adenosine triphosphate, HMP hexosemonophosphate, HDP hexosediphosphate, G-1-P glucose-1-phosphate, G-6-P glucose-6-phosphate, F-6-P fructose-6-phosphate, TCA trichloroacetic acid, Cn creatinine, Cr creatine, PGM phosphoglucomutase, G-1-6-P glucose-1-6-diphosphate.

TABLE I. Composition of Diet II.

| | | |
|---|---------------|-------------------------|
| 10 kg | Casein | (Vit. extracted) |
| 10 | Lard | (Molecularly distilled) |
| 28 | Sucrose | (Commercial) |
| 2 | Salt mixture* | |
| 523 g | Vitamin† | |
| 5 kg | Alphacel | |
| Vit. E orally, 50 mg twice weekly to controls | | |

* Hubbell, *et al.*(10).

† Vitamin Fortification Mix, Nutritional Biochemicals Corp.

they never reached the "state 3" described by Mackenzie and McCollum(4). Muscles had the waxy appearance of dystrophic muscle, with fibers varying greatly in size; but did not show complete wasting of severe muscular dystrophy. The explanation for the mild effects obtained with these diets is not clear to us, but it is not due to small amounts of Vit. E in the dietary ingredients, since the same materials used in a combination indicated by Young and Dinning(5), produce a very serious muscular dystrophy with deaths of animals after 3 to 5 weeks on the diet. Animals were sacrificed in pairs, one deficient in and one supplemented with Vit. E. Those on Mason-Harris diet were sacrificed at intervals between 7 and 42 days. The rabbits on Diet II were studied at about 60 days after starting on diet. *Enzyme preparations.* Animals were stunned by blow on head and exsanguinated. Back muscles were excised immediately; muscle of one side was used to prepare muscle strips, while the other was used to prepare an extract. A sample usually weighing a gram, was ground in a cooled mortar and extracted with 3 volumes of cold salt solution of 0.1 M KCl, 0.25 M NaHCO₃, and 0.01 M MgSO₄. The suspension was centrifuged at 0°C and the supernatant used as enzyme source. For some experiments it was desirable to dialyze the enzyme preparation. Dialysis was performed in the cold for 1½ to 3 hours in the salt solution to which 16 mg % of cysteine was added and then diluted with 1 vol. of distilled water. The dialysis medium was changed each half hour. *Enzyme determinations: PO₄ transfer from C-P to HMP.* Preliminary determinations showed that transference of phosphate to HMP was faster when G-1-P was used as co-substrate

rather than G-6-P or F-6-P. Consequently, G-1-P was used as standard acceptor. The reaction system consisted of 1.3 μM of C-P (Sigma Ca-C-P converted to Na-C-P), 3 μM of Na-G-1-P (Schwarz), 2 μM cysteine, and 0.01 or 0.02 ml of enzyme preparation. Total volume was 0.25 ml, pH 7.5, and incubated 10 minutes at 30°C. Reaction was stopped with 0.3 ml of 10% TCA and precipitated protein removed by centrifugation. Phosphate was determined by the Fiske and Subbarow method. Control experiments were identical except that C-P was added after enzyme precipitation by TCA. Results were calculated from the difference in acid-labile phosphate between experimental and control. Between 0.01 and 0.07 ml of 1:3 extract, the transference of P is a linear function of amount of muscle extract. In all experiments the effect of added ATP (Na₂ATP Pabst Laboratories) at a concentration of 0.1 μM was also determined. *Creatinine production.* The same system employed in determination of phosphate transfer was used except that the reaction was incubated 15 minutes at 30°C, stopped by addition of 1 ml of alkaline picrate (15 ml of 1% picric acid plus 1 ml of 4N NaOH), diluted with distilled water to 3 ml, and measured for absorption in Beckman D. U. spectrophotometer at 520 mμ. C-P was added to incubated control immediately after denaturation of protein by alkaline picrate. Readings were taken at 20 and 30 minutes and extrapolated to zero time to correct for conversion of creatine to creatinine in alkaline medium. *Phosphoglucumutase reaction.* A modification of the method of Cardini, *et al.*(9), was used. The reaction mixture consisted of 3 μM of G-1-P, 1 μM of MgSO₄, 1 μM cysteine, and 0.02 ml of muscle extract diluted 1:20 or 1:30 with cold distilled water. Final volume was 0.25 ml, pH 7.5. Incubation was 15 minutes at 30°C. Formation of G-6-P was measured by reducing power according to the Somogyi-Nelson method(6). *Glycolysis.* Rate of glycolysis was determined manometrically at 30°C. Strips of muscle fibers were dissected in the cold, from rabbit back muscle without cross-cutting of fibers. Strips were selected for uniformity in diameter (approximately 0.5

TABLE III. HPO_4^- Transferred and Sugar Esters Formed in Different Conditions.

Conditions for PGM reaction: 1 μM mg, 3 μM G-1-P, 0.02 ml dialyzed muscle extract; incubated 10 min., 35°C. Conditions for measurement of fructose formed and HPO_4^- transferred: 3 μM substrate, 1.5 μM C-P, 0.02 ml dialyzed muscle extract; incubated 10 min., 25°C.

| Substrate | μM ATP added | μM HPO_4^- transferred | μM G-1-6-P* formed | μM G-6-P + F-6-P formed | μM fructose formed |
|-----------|-------------------------|--|-------------------------------|------------------------------------|-------------------------------|
| G-1-P | .0 | .69 | | 2.0 | |
| | .025 | .58 | | 1.09 | .849 |
| | .050 | .37 | | .72 | |
| | .100 | .04 | | .41 | .216 |
| G-6-P | .0 | .59 | | | .503 |
| | .100 | 1.07 | | | .716 |
| G-1-P | .0 | .923 | | | .690 |
| | .100 | .290 | | | .235 |
| G-1-P | .0 | .51 | .015 | | |
| | .0 | .88 | .022 | | |

* Determined by enzymatic method of Cardini, *et al.*(9).

mm) of fibers. Incubation was performed in 7 ml flasks containing 1 g of muscle strips, 1.8 ml of Krebs-Ringer bicarbonate buffer, 0.2 ml of 0.2 M glucose in side bulb, and 0.2 ml of 20% KOH in center well. Vessels were gassed with 5% CO_2 - 95% N_2 for 10 minutes. After 15 minute equilibration period, glucose solution was tipped into the main chamber. CO_2 production was measured 30 minutes.

Results. Method to measure phosphate transfer: a) *Selection of acceptor of phosphate.* F-6-P is presumed to be the acceptor of phosphate in reaction (II), however, (Table II) our experience has been that the reaction is consistently faster in presence of G-1-P. It is not known whether the preparation of F-6-P has an inhibitory impurity, or if the F-6-P itself is an inhibitor at rather high concentration that must be used for accurate determination. To corroborate that in reaction II the HMP directly concerned is F-6-P, fructose esters found as end products of reaction were determined (Table III) by

the method of Roe(7). Micromoles of fructose formed, when compared to micromoles of phosphate transferred, were sometimes less than would be expected if the reaction is terminated at fructose-1-6-diphosphate. Loss of fructose esters may have resulted from further steps in glycolysis. The known mechanisms which could mediate an increase in total phosphate transferred via glucose esters are the reactions by which the phosphate of C-P is transferred to G-1-P to form G-1-6-P(1,8). Formation of G-1-6-P can be measured specifically by its coenzymatic property(9), and since only about 3% of total phosphate transferred appears to be this compound (Table III), it was concluded that the role of G-1-P as a direct phosphate acceptor is a very minor one. b) *Participation of the adenylic system in the reaction.* An adenylic derivative is not added to the reaction in the system for measurement of phosphate transfer from C-P. Preliminary experiments showed that addition of ATP in concentration of 0.1 μM /0.25 ml resulted in slight activation, no effect, or sometimes a small inhibition. At higher concentrations of ATP, inhibition resulted uniformly. This variability in effect of ATP may be a function of quantity of endogenous ATP present in individual enzyme extracts. To study the effect more precisely, it was necessary to dialyze the enzyme extract. Phosphate transfer was stimulated by ATP at concentrations up to 0.1 μM when dialyzed extracts were used (Table IV). c) *Inhibition of PGM by*

TABLE II. Comparison of Rate of HPO_4^- Transfer from C-P to HMPs by Non-dialyzed Muscle Extract. Conditions: As stated under *Methods*.

| Phosphate acceptor, 3 μM | μM HPO_4^- transferred | | | |
|-------------------------------------|--|--------------|-------|-------|
| | 3 | Time in min. | | |
| | | 6 | 9 | 12 |
| G-1-P | .408 | .914 | 1.078 | 1.112 |
| G-6-P | .182 | .482 | .816 | .854 |
| F-6-P | .246 | .408 | .638 | .768 |

TABLE IV. Effect of ATP on HPO_4^- Transfer from C-P to HMP. Conditions: As stated under *Methods*.

| Phosphate acceptor, 3 μM | μM ATP added | μM HPO_4^- transferred | |
|-------------------------------------|-------------------------|--|---------------------------------|
| | | Dialyzed enzyme preparation | Non-dialyzed enzyme preparation |
| G-1-P | .0 | | |
| | .001 | .07 | |
| | .01 | .379 | |
| | .1 | .842 | |
| | 1.0 | .600 | |
| G-1-P | .0 | .154 | .558 |
| | .2 | .485 | .218 |
| G-6-P | .0 | | .489 |
| | .2 | | .479 |
| F-6-P | .0 | .285 | .445 |
| | .2 | .520 | .445 |

ATP. The inhibitory effect of ATP observed above was probably due to inhibition of the phosphoglucomutase reaction. Table III shows that with G-1-P as acceptor, there is a decrease in formation of G-6-P, fructose phosphates, and in activity of phosphate transfer when the concentration of ATP is increased. When G-6-P is the acceptor both production of fructose phosphate (as determined by the resorcinol reaction of Roe) and transference of phosphate from creatine-phosphate are enhanced in presence of 0.1 μM of ATP as compared to no ATP. With G-1-P as co-substrate, this level of ATP inhibits both fructose and G-6-P formation and also phosphate transfer. However, G-1-P still appears to be the best acceptor of phosphate from C-P in our reaction system (Tables III and IV).

Effect of tocopherol deficiency on enzyme activities of muscle extracts. The first change in enzyme activity was observed in rabbits fed the Mason-Harris diet for 25 days (Fig. 1). At this time, rate of glycolysis of glucose by muscle strips of Vit. E-deficient animals (Mean 4.30 $\mu\text{M}/\text{CO}_2/\text{g}/30''$) begins to increase as compared to that of tocopherol-supplemented controls (3.02 $\mu\text{M}/\text{CO}_2/\text{g}/30''$). Glycolysis remains elevated to about 40 days on the diet, after which there is a trend to return to normal. Concurrent with changes in glycolysis, transfer of phosphate from C-P to HMP by Vit. E-deficient animals decreases (Mean 0.33 μM HPO_4^-) as com-

pared to that of controls (Mean 0.45 μM HPO_4^-) and shows no tendency to return to normal value. Production of creatinine is also diminished in Vit. E-deficient animals (Means: +E = 0.185 μM , -E = 0.132 μM). Differences observed in the above systems are significant at the 1% level when submitted to the Fisher F test. Activity of the phosphoglucomutase reaction did not differ significantly among experimental and control animals (Means: μM G-6-P, +E .457 μM , -E .421 μM).

Animals on Diet II were studied between 60 and 70 days on the diet. Data from deficient animals compared with supplemented animals (Table V) show no significant differences in glycolysis between experimental and control animals, confirming the apparent return to normal of values observed with the Mason-Harris diet. The transfer of phosphate, however, remains significantly impaired in experimental animals and the decrease from normal (27%) is essentially as observed with the Mason-Harris diet (28%). Creatinine production is significantly diminished. Although the mean for phosphoglucomutase activity for Vit. E-deficient animals is lower it does not represent a significant difference from the controls.

Effect of ATP on transfer of HPO_4^- from C-P by muscle extracts. In conjunction with phosphate transfer measurements, parallel experiments were done in which the effect of addition of 0.1 μM of ATP was tested (Table IV). Addition of ATP decreases transfer of HPO_4^- of animals on the Mason-Harris diet. The decrease is significant for both supplemented and deficient animals. In the same reaction system but using muscle extracts from animals fed Diet II, ATP does not lower the mean for phosphate transfer significantly.

Discussion. The results presented show that there is impairment of the phosphate transfer from C-P to the HMP chain. Since neither glycolysis in general, nor phosphoglucomutase reaction are decreased, this seems to represent a rather specific effect on the system that normally phosphorylates and dephosphorylates creatine phosphate. This indicates that reaction II is also functioning

TABLE V. Enzyme Activities of Muscle Extracts from Rabbits on Diet II.*

| Diet | No. of animals | Glycolysis, $\mu\text{M CO}_2/\text{g}$ /30 min. | HPO ₄ transfer | | |
|---------------------|----------------|--|---------------------------|--|------------------------------|
| | | | from C-P to HMP | Cn formed from C-P μM | PGM reaction G-6-P formed |
| Deficient in vit. E | 12 | 8.15 (None) | .411 (Between 1-5%) | .119 (1%) | .264 (None) |
| Complete | 11 | 8.00 | .576 | .168 | .327 |

* Numbers in parentheses represent significance level of value obtained for F when activities of deficient animals are compared with those on a complete diet.

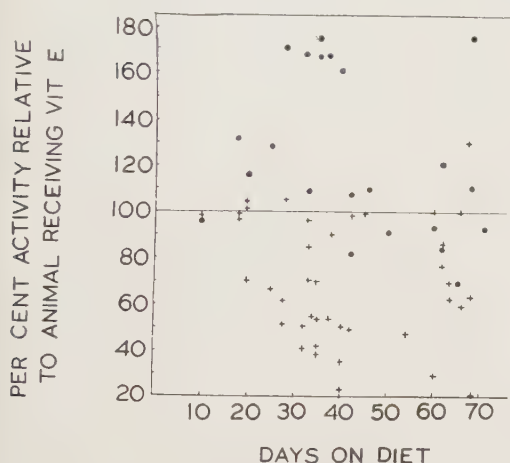


FIG. 1. Phosphate transfer by muscle extracts (+) and glycolysis by muscle strips (O) of rabbits fed the Mason and Harris diet for various lengths of time. Values are expressed as percent of the same activities of control animals.

normally, since a substantial decrease in activity here would diminish glycolysis. It is known that creatine is stored in muscle mainly as C-P and impairment of reaction I should result in decreased uptake of creatine formed by other organs. To maintain steady concentration of total creatine, this uptake has to be in equilibrium with rate of decay of creatine to creatinine. A decrease in rate of reaction I presents a possible explanation for creatinuria which is produced in tocopherol-deficient animals before other symptoms of muscle wasting and weakness. The role of tocopherol, or perhaps a derivative in reaction III, for which known co-factors are ATP and SH, is probably not that of co-factor. Also the possibility can be discarded that decrease of ATP accounts for decreased rate of phosphate transfer, since addition of ATP to the reaction system was not found to stimulate activity. Therefore, the enzyme itself re-

mains the most likely decreased component.

That there is a specific impairment of this enzymatic system relative to others studied in this work, makes it plausible to suggest that the severity of Vit. E deficiency on the muscle could be related to the requirement of muscle for unusually high concentrations of C-P as compared to other tissues.

Summary. A method for determination of the transfer of phosphate from creatine-phosphate to the hexosemonophosphates is described. It has been found that this transference is decreased in the muscle of rabbits fed tocopherol-deficient diets at a time when phosphoglucomutase activity and glycolysis are normal. The decrease in transference is not corrected by addition of ATP. ATP added at concentrations above 0.004 M inhibits phosphoglucomutase.

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Correlation of Rf Values and Distribution Coefficients in Amino Acid Paper Chromatography. (23692)

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Paper chromatography, as an analytical procedure, is particularly useful in analysis of biological materials in which the available samples are often extremely small. The method is quite popular for analysis of mixtures of amino acids, but the details of the procedures used by various investigators vary considerably(1-11). As part of a rather extensive program of investigation in regard to the various factors controlling the development of the chromatogram, in the analysis of amino acid mixtures, we have determined the relationship of solubility, of amino acids in phenol and water mixtures, to the corresponding Rf values obtained by paper chromatography(12), using phenol as the developing agent.

Methods. Redistilled phenol was emulsified with buffered water solutions of individual amino acids under controlled conditions of temperature. The layers were allowed to separate and the concentration of amino acid in each layer determined by the Kjeldahl Method. Distribution coefficients (Kd) were calculated by determining the ratio of Nitrogen concentration in the water phase to Nitrogen concentration in the phenol phase, at temperatures of 20, 25 and 30°C. A scatter diagram (Fig. 1) was prepared and the mathematical relationship between the 2 groups of data determined to be $R_f = 0.781 - .474 \log K_d - .433 (\log K_d)^2 + .259 (\log K_d)^3$. Theoretical Rf values were calculated and compared with published(12) Rf values (Table I). The Pearson Product Moment Coefficient of Correlation, *r*, was determined to be 0.965 (which is significant at the 1% level for 77 degrees of freedom).

Discussion. The data presented indicate that a relationship exists between distribution coefficients, of amino acids in the 2 phases of a phenol-water system, and Rf values, in amino acid paper chromatography, using the same phenol-water system as the developing agent. This would tend to support the hy-

pothesis that, in paper chromatography, the paper acted as an inert mechanical support of the water or stationary phase and that the amino acid spot moved along the paper with the flow of developing agent by a process of continuous, or multiple, extraction and re-extraction. Several variables should be considered and standardized.

There probably is a "water in phenol" concentration gradient which would affect very greatly the reextraction process and the movement of the amino acid spot, and thus, the Rf value. This concentration gradient would be, in turn, affected by temperature, state of initial saturation of the phenol and the humidity of the air surrounding the strips. It would seem, therefore, that the relative amounts of water and phenol should be carefully standardized, that the humidity of the cabinet should be controlled and that the distance, on the paper strips to which the developing solution progresses, should be constant.

Summary. The distribution coefficients of amino acids between the 2 phases of a phenol-water mixture have been determined at various H⁺ concentrations, and at temperatures of 20°, 25°, and 30°C. Rf values cal-

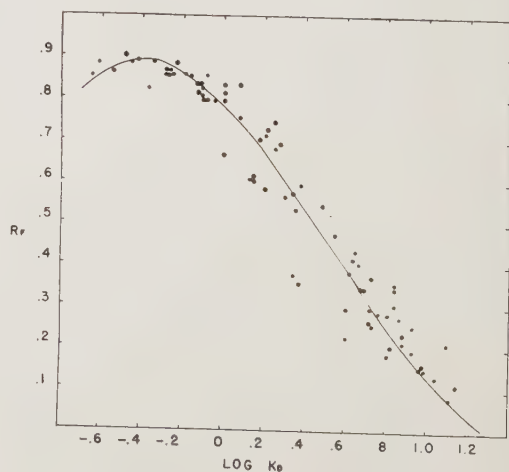


FIG. 1. Comparison of Rf (published) with log Kd (determined).

TABLE I. Comparison of Kd (Determined) with Rf (Calculated) and Rf (Published).

| Amino acid | pH | Kd at °C | | | | Rf calc. at °C | | | | Rf published (McFarren) |
|----------------|------|----------|------|------|------|----------------|----|----|----|----------------------------|
| | | 20 | 25 | 30 | 22 | (× 100) | | | | (× 100) |
| | | | | | | 20 | 25 | 30 | 22 | 22 |
| Alanine | 2.0 | 4.5 | 4.4 | 4.3 | 4.5 | 36 | 37 | 38 | 36 | 43 |
| | 6.2 | 4.7 | 4.0 | 3.4 | 4.4 | 34 | 40 | 44 | 36 | 41 |
| | 10.0 | 3.4 | 3.8 | 3.4 | 3.6 | 44 | 41 | 44 | 43 | 47 |
| | 11.2 | 4.5 | 3.8 | 3.9 | 4.2 | 36 | 41 | 40 | 38 | 38 |
| Arginine | 2.0 | 7.4 | 6.8 | 5.7 | 7.2 | 21 | 24 | 28 | 22 | 35 |
| | 6.2 | 4.1 | 4.2 | 4.3 | 4.1 | 39 | 38 | 38 | 38 | 29 |
| | 10.0 | .71 | .74 | .87 | .72 | 84 | 84 | 81 | 84 | 87 |
| | 11.2 | 1.0 | 1.2 | 1.4 | 1 | 78 | 74 | 70 | 78 | 66 |
| Aspartic acid | 2.0 | 7.9 | 7.6 | 6.7 | 7.8 | 19 | 20 | 24 | 20 | 21 |
| | 6.2 | 9.9 | 9.3 | 8.8 | 9.7 | 14 | 15 | 16 | 14 | 15 |
| | 10.0 | 14.3 | 14.5 | 15.3 | 14.4 | 8 | 8 | 7 | 7 | 11 |
| | 11.2 | 14.9 | 10.9 | 13.2 | 13.3 | 5 | 12 | 7 | 7 | 8 |
| Cystine | 2.0 | 11.9 | 10.0 | 10.0 | 11.2 | 10 | 13 | 13 | 11 | 13 |
| | 6.2 | | | | | | | | | |
| | 10.0 | 5.3 | 5.5 | 5.5 | 4.1 | 31 | 29 | 29 | 30 | 29 |
| | 11.2 | 4.6 | 5.4 | 4.8 | 4.9 | 34 | 30 | 34 | 33 | 22 |
| Glutamic acid | 2.0 | 7.1 | 6.7 | 6.6 | 7.0 | 22 | 24 | 24 | 22 | 30 |
| | 6.2 | 6.4 | 7.0 | 7.0 | 6.6 | 25 | 23 | 23 | 24 | 28 |
| | 10.0 | 8.2 | 9.5 | 9.2 | 8.7 | 18 | 15 | 15 | 17 | 19 |
| | 11.2 | 9.1 | 10.5 | 10.5 | 9.6 | 16 | 13 | 13 | 15 | 16 |
| Glycine | 2.0 | 9.2 | 8.4 | 8.5 | 8.9 | 15 | 18 | 17 | 16 | 25 |
| | 6.2 | 5.2 | 5.8 | 5.8 | 5.4 | 32 | 28 | 28 | 30 | 26 |
| | 10.0 | 5.2 | 4.6 | 5.2 | 5.0 | 32 | 34 | 32 | 33 | 34 |
| | 11.2 | 5.2 | 6.0 | 5.2 | 5.5 | 32 | 27 | 32 | 30 | 25 |
| Histidine | 2.0 | 7.1 | 8.2 | 6.5 | 7.5 | 22 | 18 | 15 | 21 | 27 |
| | 6.2 | 2.4 | 2.3 | 2.4 | 2.4 | 55 | 56 | 55 | 55 | 35 |
| | 10.0 | 1.0 | 1.1 | 1.2 | 1.0 | 78 | 76 | 74 | 78 | 68 |
| | 11.2 | 1.1 | 1.2 | 1.2 | 1.1 | 76 | 74 | 74 | 76 | 68 |
| Hydroxyproline | 2.0 | 3.1 | 3.1 | 2.8 | 3.1 | 47 | 47 | 51 | 47 | 54 |
| | 6.2 | 2.4 | 2.2 | 2.4 | 2.3 | 55 | 58 | 55 | 56 | 53 |
| | 10.0 | 2.5 | 2.1 | 2.1 | 2.4 | 54 | 59 | 59 | 55 | 59 |
| | 11.2 | 2.1 | 2.4 | 2.4 | 2.2 | 59 | 55 | 55 | 58 | 57 |
| Isoleucine | 2.0 | 1.0 | 1.0 | 1.0 | 1 | 78 | 78 | 78 | 78 | 81 |
| | 6.2 | .78 | .83 | .86 | .79 | 83 | 82 | 81 | 83 | 82 |
| | 10.0 | .74 | .74 | .74 | .74 | 83 | 83 | 83 | 83 | 81 |
| | 11.2 | .74 | .80 | .77 | .76 | 83 | 82 | 82 | 82 | 83 |
| Leucine | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 | 78 | 78 | 78 | 78 | 79 |
| | 6.2 | .75 | .82 | .79 | .78 | 83 | 82 | 82 | 83 | 85 |
| | 10.0 | 1.2 | .75 | .69 | 1.0 | 74 | 83 | 85 | 78 | 83 |
| | 11.2 | .73 | .71 | .72 | .73 | 85 | 84 | 84 | 84 | 83 |
| Lysine | 2.0 | 12.4 | 14.1 | 12.1 | 13.1 | 9 | 7 | 9 | 8 | 21 |
| | 6.2 | 10.5 | 8.7 | 8.5 | 9.8 | 12 | 17 | 17 | 14 | 15 |
| | 10.0 | 1.8 | 1.8 | 1.8 | 1.8 | 63 | 63 | 63 | 63 | 74 |
| | 11.2 | 2.1 | 2.5 | 3.9 | 2.2 | 58 | 54 | 40 | 57 | 37 |
| Methionine | 2.0 | 1.2 | 1.2 | 1.2 | 1.2 | 74 | 74 | 74 | 74 | 75 |
| | 6.2 | .86 | .97 | 1.0 | .90 | 81 | 79 | 78 | 80 | 79 |
| | 10.0 | .80 | .84 | .88 | .82 | 82 | 81 | 80 | 82 | 79 |
| | 11.2 | .79 | .80 | .85 | .79 | 83 | 82 | 81 | 82 | 79 |
| Phenylalanine | 2.0 | .50 | .55 | .53 | .52 | 88 | 87 | 87 | 88 | 85 |
| | 6.2 | .36 | .39 | .39 | .37 | 88 | 89 | 89 | 89 | 89 |
| | 10.0 | .34 | .37 | .41 | .35 | 88 | 89 | 89 | 88 | 88 |
| | 11.2 | .32 | .34 | .34 | .33 | 88 | 88 | 88 | 88 | 90 |
| Proline | 2.0 | 1.0 | .93 | 1.0 | .97 | 78 | 79 | 78 | 78 | 80 |
| | 6.2 | .69 | .65 | .81 | .68 | 85 | 86 | 82 | 85 | 85 |
| | 10.0 | .64 | .74 | .73 | .68 | 85 | 83 | 83 | 85 | 84 |
| | 11.2 | .62 | .67 | .75 | .64 | 86 | 85 | 83 | 86 | 85 |

TABLE I (continued).

| Amino acid | pH | Kd at °C | | | | Rf calc. at °C | | | | Rf published (McFarren) |
|-------------|------|----------|-----|-----|-----|----------------|----|----|----|----------------------------|
| | | 20 | 25 | 30 | 22 | (× 100) | | | | (× 100) |
| Serine | 2.0 | 8.1 | 7.2 | 7.8 | 7.8 | 19 | 22 | 20 | 20 | 23 |
| | 6.2 | 6.5 | 7.2 | 7.0 | 6.8 | 25 | 22 | 22 | 23 | 20 |
| | 10.0 | 5.8 | 6.1 | 4.5 | 5.9 | 27 | 26 | 35 | 27 | 28 |
| | 11.2 | 6.6 | 6.5 | 6.2 | 6.6 | 24 | 24 | 26 | 24 | 18 |
| Threonine | 2.0 | 7.1 | 6.7 | 6.0 | 7.0 | 22 | 23 | 27 | 22 | 35 |
| | 6.2 | 5.6 | 5.0 | 5.0 | 5.4 | 29 | 33 | 33 | 30 | 37 |
| | 10.0 | 4.7 | 4.5 | 3.9 | 4.6 | 35 | 38 | 40 | 35 | 40 |
| | 11.2 | 5.0 | 4.8 | 4.6 | 4.9 | 33 | 34 | 35 | 33 | 34 |
| Tyrosine | 2.0 | 2.0 | 2.0 | 1.8 | 2.0 | 60 | 60 | 64 | 60 | 56 |
| | 6.2 | 1.4 | 1.4 | 1.5 | 1.4 | 70 | 70 | 68 | 70 | 60 |
| | 10.0 | 1.4 | 1.4 | 1.3 | 1.4 | 70 | 70 | 72 | 70 | 61 |
| | 11.2 | 1.4 | 1.4 | 1.4 | 1.4 | 70 | 70 | 70 | 70 | 60 |
| Tryptophane | 2.0 | .41 | .43 | .44 | .42 | 89 | 89 | 89 | 89 | 82 |
| | 6.2 | .27 | .29 | .29 | .28 | 86 | 87 | 87 | 86 | 85 |
| | 10.0 | .22 | .27 | .29 | .24 | 83 | 86 | 87 | 84 | 88 |
| | 11.2 | .22 | .23 | .24 | .22 | 83 | 84 | 84 | 83 | 85 |
| Valine | 2.0 | 2.0 | 1.7 | 1.8 | 1.9 | 60 | 65 | 63 | 62 | 69 |
| | 6.2 | 1.7 | 1.5 | 1.6 | 1.6 | 65 | 68 | 66 | 67 | 71 |
| | 10.0 | 1.5 | 1.5 | 1.5 | 1.5 | 68 | 68 | 68 | 68 | 70 |
| | 11.2 | 1.6 | 1.5 | 1.3 | 1.6 | 66 | 68 | 72 | 67 | 72 |

culated from each of these distribution coefficients have been correlated by statistical methods with corresponding Rf values as measured by paper chromatography and a significant relationship has been demonstrated.

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Host-Virus Relationships in Rous Sarcoma Tissues *in vitro*. I. Growth of Extraneous Viruses.* (23693)

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Considerable experimental work has been recorded on the growth of viruses in neoplastic tissues(1). For such investigations most workers have utilized transplantable tis-

sues of tumors not known to be caused by viruses, while few studies have been made of the growth of extraneous viruses in the cells of virus-induced tumors. In investigating the biological properties of a tumor-inducing virus and the properties of the malignant tissue it produces, the ability of Rous sarcoma tissue

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(2) to support the growth of several extraneous animal viruses has been studied. The Rous sarcoma tissue is uniquely suited for such studies, for it is of proven malignancy; it is a consequence of virus infection of normal connective tissue elements(3); and recent evidence indicates that all Rous tumor cells contain and slowly produce the Rous virus(4). Thus, by employing this particular tissue, it has been possible to study the capacity of a malignant tissue already infected with a cancer virus to support the growth of certain non-neoplastic viruses. The ability of normal chick embryo and adult chick fibroblastic tissues to support the proliferation of the same infecting viral agents has also been studied. It was anticipated that the findings from such studies might provide valuable information of several varieties. The studies of the attempted superinfection of the Rous cells were of interest for the information they might give regarding possible demonstration of an interference phenomenon or of dual virus infection of tumor cells. This information in turn might elucidate certain biological properties of the Rous sarcoma virus and thereby provide some insight as to the relationship of this virus to nonneoplastic viruses. Because of the biological and morphological relationships of the embryo and adult chick fibroblastic tissues to the chick-derived Rous sarcoma tissue, the studies of the virus proliferation in these related tissues were of interest for the clues they might provide regarding similarities or differences between these 3 tissues in their reactions to viral infection.

The results indicate that of 4 viruses capable of proliferation in chick embryo fibroblastic tissue *in vitro* (Newcastle Disease virus (NDV), the NWS strain of influenza A virus (NWS), vaccinia virus and psittacosis virus), only NDV and vaccinia virus were able to reproduce in cultures of adult chick fibroblastic tissue and of Rous sarcoma tissue.

Materials and methods. Viruses. The Rous sarcoma virus used for the induction of tumors was the partially purified CT-581 preparation(5) kindly provided by Dr. W. R. Bryan. All viruses used were stored sealed in glass in a dry-ice freezer. The 6BC strain of psittacosis virus used was passed in eggs by

the yolk sac route, and a uniform source of the agent was obtained by methods described previously(11). The strain of vaccine virus employed was that used by the New York City Board of Health. To obtain a fairly uniform source of the virus, 12-day embryonated eggs were infected by the CAM route. After 72 hours' incubation, the membranes were removed, pooled, homogenized, centrifuged and diluted in infusion broth to make a 20% suspension by weight. The B strain of NDV and the NWS strain of influenza virus were prepared by harvesting the infected allantoic fluids 48 hours after inoculation of eggs, centrifuging the materials, and removing the supernatant fluids. *Virus titration.* The presence of Rous sarcoma virus in tumor cell cultures was determined by the ability of the material tested to produce characteristic lesions in young chicks. 0.20 ml of the homogenates of the cultures prepared as below were inoculated into the wing web of 6 to 8 white leghorn chicks under 10 days of age according to the method of Bryan *et al.*(5). In some cases, white leghorn chicks were inoculated intracerebrally with 0.05 ml of the homogenate as described by Groupé and co-workers (6). In determining the amount of psittacosis virus in seed stock or in culture materials, the single dilution technic of Golub was employed(7). In titration of vaccinia-infected materials, the 50% lethal endpoint was calculated according to the method of Reed and Muench(8) using the method described previously(9a,b). Stock and experimental fluids infected with NDV and influenza virus were diluted in serial 10-fold dilutions in chilled beef infusion broth and four 9-day-old embryos were inoculated allantoically with 0.20 ml of each of the dilutions. After 48 hours' incubation at 35 to 36°C, 0.5 ml of allantoic fluid was removed from each egg and the presence of virus determined by testing for hemagglutinins by the method of Granoff and Henle(10). The 50% infectivity endpoint was calculated. *Culture Media.* The culture media employed were: A. In chick embryo fibroblastic cultures, horse serum 23.0%, Lactalbumin hydrolysate 0.8%, Hanks' balanced salt sol. (BSS) 76.2%. B. In adult chick fibroblast and Rous sar-

TABLE I. Growth of NWS Influenza Virus.

| Tissue | Inoculum | Virus content | | | | | |
|----------------|---|--|--|--------------------------|--------------------------|--------------------------|---|
| | | 6 hr | 2nd washing at 6 hr | 2 days | 4 days | 6 days | 8 days |
| Chick embryo | 10 ^{4.5*} | F = 10 ^{3.25} | 10 ^{2.17} | F = 10 ^{4.76} | F = 10 ⁵ | F = 10 ⁵ | F = 10 ^{5.76} T = 10 ⁶ |
| Adult chick | 10 ^{4.75} | F = 10 ⁴ | 10 ^{1.75} | F = 0 | F = 0 | F = 0 | F = 0 T = 0 |
| Rous sarcoma | 10 ^{4.76} 10 ^{7.0} | F = 10 ⁴ F = 10 ⁶ | 10 ^{2.4} 10 ^{5.0} | F = 0 T = 0 " " T = 0 | F = 0 T = 0 " " T = 0 | F = 0 T = 0 " " T = 0 | F = 0 T = 0 " " T = 0 |
| Virus controls | 10 ^{4.5} | 10 ⁴ | — | 0 | 0 | 0 | 0 |
| Tissue " | 0 | 0 | — | F = 0 T = 0 | F = 0 T = 0 | — | F = 0 T = 0 |

* LD₅₀.

F = fluids, T = tissues, — = not tested.

coma cultures, horse serum 40.0%, Lactalbumin hydrolysate 0.8%, BSS 59.2%. Media were buffered to pH 7.4 with 4% sodium bicarbonate and contained 100 units/ml of penicillin and 50 gamma/ml of streptomycin. No antibiotics were used in the psittacosis cultures. *Tissues.* The Rous sarcoma tissue used was taken from tumors produced in the wing web of white leghorn chicks by the subcutaneous inoculation of dilutions of the standard stock preparation in chicks under 10 days old. Tissue was removed for *in vitro* culture 10 to 14 days after inoculation from tumors which were rapidly developing and not necrotic. The malignant appearing tissue was dissected, efforts being made to avoid the inclusion of nonmalignant muscle and/or connective tissue. The adult chick tissue used was the skin, connective tissue and muscle dissected as above from the wing web area of 20- to 30-day-old white leghorn chicks. The embryo fibroblastic tissue employed was the skin, connective tissue and muscle dissected from the wings and legs of 12-day-old embryos. *Culture technic.* The tissue cultures were prepared as described previously (11), using fragments of tissues planted under celophane discs in 10-ml Erlenmeyer flasks. The initial medium was completely removed after 24 hours' incubation at 36 to 37°C, and the tissue washed once with BSS. Each of 4 cultures was infected with a particular virus and after 6 hours' incubation at 36 to 37°C, the fluids were completely removed, pooled, centrifuged for 5 minutes at 2,000 rpm and the supernatant fluid taken for the determination of virus content. The tissue was washed

twice with 2 ml of BSS, the second washing being prepared as above for virus titration. Thereafter, at 2-day intervals, the culture fluids were completely removed and prepared for titration. Each time, as the medium was removed, the tissues were washed once with BSS and fresh medium replaced. The fluids were titrated for the presence of both the exogenous virus and Rous sarcoma virus in the case of the Rous sarcoma cultures. In some experiments, the tissue from one flask was removed at the time of harvest of the fluid medium and was prepared for determination of virus content. In all experiments the tissues of the flasks remaining at the end of the 8-day experimental period were pooled and prepared for virus titration. The tissue was ground in a mortar with alundum in 1 ml of BSS. The homogenate was then centrifuged for 5 minutes at 2,000 rpm and the supernatant removed for titration. Since these homogenates were tested for activity of the Rous sarcoma virus as well as the extraneous virus, careful microscopic examination was made of the material to assure that intact cells were not present.

Controls. Virus controls to determine thermal inactivation of the individual viruses were prepared by inoculating the same quantity of virus used in the experimental protocol into 10-ml Erlenmeyer flasks containing an identical quantity of the appropriate medium. These tissue-free cultures were incubated at 36 to 37°C and at periodic intervals aliquots were taken for the estimation of concentration of active virus.

Results. The results of experiments to de-

TABLE II. Growth of Psittacosis Virus.

| Tissue | Inoculum | Virus content | | | | | |
|---------------|-------------|----------------|---------------------|-----------------|----------------|-----------------|----------------------------------|
| | | 6 hr | 2nd washing at 6 hr | 2 days | 4 days | 6 days | 8 days |
| Chick embryo | $10^{4.8*}$ | F = $10^{3.3}$ | $10^{2.45}$ | F = $10^{3.75}$ | F = $10^{5.5}$ | F = $10^{5.78}$ | F = $10^{5.2}$ T = $10^{5.8}$ |
| Adult chick | $10^{4.7}$ | F = $10^{3.1}$ | $10^{2.5}$ | F = 0 | F = 0 | F = 0 | F = 0 T = 0 |
| Rous sarcoma | 10^5 | F = $10^{3.9}$ | $10^{2.48}$ | " | " | " | " |
| Virus control | 10^5 | $10^{3.15}$ | — | 0 | 0 | 0 | 0 |
| Tissue " | 0 | 0 | — | 0 | — | 0 | — |

* LD₅₀.

F = fluids, T = tissues, — = not tested.

termine the ability of the NWS type of influenza A virus to multiply in the embryo, adult and tumor tissues are summarized in Table I. Active multiplication of NWS was evident when the embryo fibroblastic tissue was used as the host tissue. During the 6-hour period allowed for attachment of the virus, concentration of the NWS recoverable from the fluids of the cultures diminished markedly. However, at 2-day intervals thereafter the concentration of the extracellular virus steadily increased, in contradistinction to that in the virus control cultures where no virus was demonstrable at any examination after the initial 6-hour titration. The only examination of the virus content of the tissues was made on the 8th day of infection and a large amount of intracellular virus was demonstrated.

In contrast to these findings, there was no NWS virus demonstrable in either the fluids or the tissues of the adult chick or Rous tumor cultures at any time later than 6 hours following the inoculation of the virus. And in the case of the tumor tissue, these findings were independent of the concentration of the NWS inoculum. Such results clearly indicate that there was no proliferation of the NWS virus in these cultures.

The tissues of both types of cultures, however, were metabolically active, as evidenced by the drop in the pH of the culture medium, and the finding of active Rous sarcoma virus in both fluids and tissue of the tumor cultures indicated that virus was present. Furthermore, no hemagglutination-inhibiting substances were found in either the medium or extracts of the tissues which might have masked the NWS virus. Thus, the inability

of the NWS virus to multiply in the cultures of the adult chick and Rous sarcoma tissues *in vitro* is due to some direct relationship between the cells and the virus and not to extraneous factors.

The findings with psittacosis virus are summarized in Table II. They appear qualitatively similar to the results of the NWS studies in that there is clear evidence of psittacosis virus multiplication in the chick embryo tissue but no indication of proliferation of the virus in the adult chick or tumor tissues.

The concentration of psittacosis virus demonstrable in the medium of the embryo tissue cultures was found to increase progressively throughout the course of the experimental period with maximum titers found 6 days after infection.

No psittacosis virus was demonstrable in the fluids of the cultures of the adult chick tissue or of the Rous sarcoma tissue after the 6-hour examination, and no intracellular virus was evident in the tissues 8 days after virus inoculation. All the cultures contained viable tissue throughout the course of the experiment, and Rous sarcoma virus was present in the tumor cultures.

To insure that Rous sarcoma virus was not interfering with the growth of influenza or psittacosis viruses in the eggs used for titration, Rous virus was injected into either the allantoic or the yolk sac before injection of influenza or psittacosis virus, respectively, and no effect was observed on the subsequent growth of these two viruses, thus excluding the possibility of a viral interference reaction.

Table III summarizes the results of the experimentation with the vaccinia virus. It is

TABLE III. Growth of Vaccinia Virus.

| Tissue | Inoculum | Virus content— | | | | | |
|----------------|--------------------|------------------------|------------------------|------------------------|------------------------|------------------------|--|
| | | 6 hr | 2nd washing at 6 hr | 2 days | 4 days | 6 days | 8 days |
| Chick embryo | 10 ^{4.6*} | F = 10 ^{4.47} | 10 ^{2.91} | F = 10 ^{3.42} | F = 10 ^{5.5} | F = 10 ^{5.16} | — |
| | 10 ^{4.55} | F = 10 ^{4.21} | 10 ^{2.5} | F = 10 ^{1.03} | F = 10 ^{3.3} | F = 10 ^{4.5} | F = 10 ^{2.77} T = 10 ^{2.25} |
| Adult chick | 10 ^{4.63} | F = 10 ^{3.63} | 10 ^{2.5} | F = 10 ^{2.3} | F = 10 ^{2.65} | — | F = 10 ⁴ T = 10 ^{5.66} |
| | 10 ^{6.6} | F = 10 ^{8.25} | 10 ^{3.5} | F = 10 ^{3.20} | F = 10 ^{4.32} | F = 10 ^{5.56} | — |
| Rous sarcoma | 10 ^{4.55} | F = 10 ^{3.43} | 10 ^{2.75} | F = 10 ^{2.63} | F = 10 ^{2.0} | F = 10 ^{1.55} | F = 0 T = 10 ¹ |
| | 10 ^{5.0} | F = 10 ⁵ | 10 ⁴ | F = 10 ^{2.76} | F = 10 ^{3.4} | F = 10 ^{2.81} | — |
| Virus controls | 10 ^{4.55} | F = 10 ^{3.5} | — | F = 10 ³ | F = 10 ^{2.8} | F = 10 ^{1.76} | 0 |
| Tissue " | 0 | 0 | — | 0 | — | 0 | — |

* LD₅₀.

F = fluids, T = tissues, — = not tested.

evident that the concentration of the virus in the medium of the embryo cultures rose slowly but progressively from that present in the cultures when the unadsorbed virus was removed. Though the multiplication of the vaccinia virus was slow, it did increase in titer, while the virus in control cultures was found to lose its infectivity slowly and to disappear from the culture medium in 6 to 8 days.

In other studies using similar technics, growth curves similar to this presented for vaccinia virus in embryo skin-muscle tissue have been found in cultures of chick embryo heart, brain, gut and liver tissues (12).

The course of infection of the adult chick fibroblastic tissue by the vaccine virus was also indicative of low-grade virus multiplication. Fluids removed from the cultures at 2-day intervals demonstrated progressively increasing amounts of the virus, regardless of concentration of the original inoculum. Results similar to these have also been found in vaccinia virus infections of adult chick brain, heart, gut and liver tissues under identical *in vitro* conditions (unpublished observations).

The results with the vaccinia virus in the tumor tissue were not as clearly definable as those above. In 2 separate experiments, the concentration of the virus recovered from the cultures after 2 washings with BSS 6 hours after virus inoculation was approximately 70 to 90 times less than the inoculum. At 2-day

intervals thereafter, the concentration of the virus demonstrable in the culture fluids, completely removed for each titration, was progressively less than that obtained at the previous examination with one exception. By the 8th postinoculation day, there was no active virus in the fluids and only a small amount present in the tissues.

If one considers only that this growth pattern shows a progressive diminution in titer, comparing favorably to that of the control cultures, there is no evidence of vaccinia virus multiplication. The results could then be explained on the basis of elution and/or dilution of the virus in the cultures with continual thermal inactivation. But if one considers that the virus in the control flasks was not removed from the culture at 2, 4 or 6 days, while the fluids of the experimental tissue flasks were totally removed and titered at 48-hour intervals, with unattached virus being washed out before replenishment of the medium, it seems more likely that these results indicate a low-grade reproduction of vaccinia virus in the Rous tissue. Definitive interpretation awaits more detailed experiments, but it is probable that vaccinia virus is able to proliferate slowly in Rous sarcoma cells in which Rous sarcoma virus was demonstrable.

The results of the growth of Newcastle Disease virus in the cultures of the various tissues are summarized in Table IV and indicate multiplication of NDV in all 3 tissues, but viral reproduction was much slower in adult

TABLE IV. Growth of NDV Virus.

| Tissue | Inoculum | Virus content | | | | | |
|---------------|--------------------|--|---------------------------|---|---|---|---|
| | | 6 hr | 2nd washing at 6 hr | 2 days | 4 days | 6 days | 8 days |
| Chick embryo | 10 ^{3.5*} | 10 ^{2.75} | 10 ^{1.5} | 10 ^{3.57} | 10 ^{6.58} | 10 ^{6.26} | — |
| Adult chick | 10 ^{3.71} | — | 10 ^{1.69} | F = 10 ³ T = 10 ^{1.87} | F = 10 ⁴ T = 10 ^{3.56} | F = 10 ^{4.61} T = 10 ^{3.4} | F = 10 ⁴ T = 10 ^{4.64} |
| Rous sarcoma | 10 ^{3.5} | F = 10 ^{2.4} T = 10 ² | 10 ¹ | F = 10 ^{1.43} T = 10 ¹ | F = 10 ^{3.5} T = 10 ^{1.24} | F = 10 ^{3.73} T = 10 ^{2.5} | F = 10 ⁴ T = 10 ⁵ |
| | 10 ^{5.5} | F = 10 ³ | 10 ² | F = 10 ^{1.03} | F = 10 ² | F = 10 ^{4.26} | F = 10 ² 10 ^{6.2} |
| Virus control | 10 ^{3.71} | 10 ^{1.87} | — | 0 | 0 | 0 | 0 |
| | 10 ^{5.5} | 10 ^{3.23} | — | 0 | 0 | 0 | 0 |
| Tissue " | 0 | 0 | — | 0 | — | 0 | — |

* LD₅₀.

F = fluids, T = tissues, — = not tested.

chick and Rous sarcoma tissues than in embryo tissues. The Rous sarcoma tissues were shown to contain the causative virus.

Discussion. The results of the experiments in which embryo and normal adult chick tissue cultures were inoculated with 4 animal viruses illustrates a definite qualitative difference in susceptibility to virus infection by closely related tissues, since neither NWS nor psittacosis viruses were able to propagate in the adult tissue while multiplying freely in the embryo fibroblastic cells. NDV and vaccinia virus, on the other hand, were capable of proliferation in both types of tissues.

Such a difference in susceptibility of embryonic and adult chick tissues to infection by an A strain of influenza virus was also found by Colville and co-workers(12) while studying the factors important in the *in vitro* propagation of the PR-8 virus in chick tissues. The fact that the NWS strain of influenza A virus, which seems to multiply in a larger variety of tissues than any other influenza A virus, gives results comparable to those found by Colville *et al.* suggests that there is some uniform insusceptibility of adult chick fibroblastic cells to the A strains of influenza viruses.

That psittacosis virus should prove incapable of reproduction in adult fibroblastic tissue was surprising in light of its capacity to grow in a variety of chick embryo tissues *in vitro* and the native parasitism of closely related viruses in the chicken *in vivo*. Regarding the latter, however, although the virus has been shown to infect chickens both experi-

mentally and naturally, no report has been made of its recovery from blood-free skin-muscle tissues. This appears to be the first recorded attempt to cultivate the virus in chick fibroblastic tissue *in vitro*. Nevertheless, these experiments illustrate 2 interesting examples of an absolute difference in the susceptibility of embryo and chick tissues to virus infections *in vitro*.

In spite of the fact that all 4 viruses studied have previously been shown to multiply in malignant tissue(1) and some in virus-induced tumors, only NDV and vaccinia virus were capable of reproduction in the Rous sarcoma cultures. This represents a dual infection of the Rous cell by both the causative Rous agent and the invading NDV or vaccinia virus.

The finding that influenza and psittacosis viruses are incapable of multiplying in Rous sarcoma tissues *in vitro* is compatible with two possible explanations. First, one might attribute this failure of viral growth to a viral interference reaction exerted by the Rous sarcoma virus already present in the cells. However, the results obtained with the adult chick tissues present a second possibility, for in cultures of these 2 related types of tissue only those viruses capable of replication in the adult connective tissue and muscle elements were similarly capable of growing in the sarcomatous tissue derived from the adult fibroblastic tissue. The results obtained from the Rous sarcoma cultures indicate either that the Rous cells are themselves insusceptible to

all of the 4 agents studied and the NDV and vaccinia virus infect only normal chick cells present as "contaminants" in the cultures of the malignant tissue, or that in the transformation to malignancy the Rous cells retain certain biological properties of the normal adult chick cell, manifest here in susceptibility to virus infection, and in this respect are more like adult than embryonic tissue.

Of these possibilities, the latter seems more feasible. It is entirely reasonable to assume that certain biological elements of the normal adult chick cell are retained even after the cell is transformed into a malignant state by the Rous virus and that these properties may be intimately related to the intracellular mechanisms necessary for virus reproduction. Although it cannot be stated with certainty that the Rous cultures were "pure" in that no non-malignant chick cells were present, it can be reasonably assumed that the few such cells present could not serve adequately as host tissue for the large quantities of NDV produced. However, with vaccinia virus the low titers obtained in Rous tissue prevent exclusion for this same reason.

It appears to the authors, therefore, that under the conditions of the present studies the ability of an extraneous virus to infect the Rous sarcoma cells is dependent on certain biological properties retained by the cell from its nonmalignant predecessor and is independent of the presence and reproduction of the causative Rous agent already inhabiting the cell. If this is true, the Rous cell system differs from that reported by Ginder and Friedewald(13) in their experiments with rabbit fibroma tissues because these workers described the growth of Semliki Forest virus in the rabbit fibroma tissue both *in vivo* and *in*

vitro, but the virus failed to proliferate in normal rabbit connective tissue elements.

Summary. The ability of 4 animal viruses (NDV, NWS, vaccinia and psittacosis) to propagate in Maitland-type tissue cultures of chick embryo and adult chick fibroblastic tissues and Rous sarcoma tissue was studied and all 4 viruses were found capable of proliferation in the embryo tissue, while only NDV and vaccinia virus multiplied in the adult chick and Rous sarcoma tissue cultures. Thus, Rous sarcoma tissue appears to be more closely related to connective tissue of adult chicks than to that of chick embryos in its susceptibility to infection with certain other viruses. NDV and Rous sarcoma virus can produce a dual infection of cells.

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Passage of Shope's Rabbit Fibroma Virus through One-Day-Old Mice.* (23694)

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The virus of fibromatosis, like the closely related virus of myxomatosis, is considered specific for rabbits in nature. It produces local lesions in cottontail rabbits (*Sylvilagus* spp.) in the eastern and midwestern United States as well as in domestic rabbits under experimental conditions. To date it has not been successfully propagated in other animals, except on the chorioallantoic membrane of chick embryos(1). Since Andrewes and Harisjades(2) achieved 30 serial passages of myxoma virus in one-day-old mice, an attempt was made to do the same with the fibroma virus. This paper is a report of our preliminary experiments.

Methods. One-day-old NIH general-purpose mice were inoculated intracerebrally with 0.01 ml of a 10% suspension (400 infectious doses) of domestic rabbit tumor tissue prepared in serum broth saline and filtered through a Swinney filter after light centrifugation (1,800 rpm for 7 minutes). The fibroma virus used was the Boerlage strain,§ shipped to the author as infectious rabbit testicle which was then passed by serial intracutaneous injections in domestic rabbits. The serum broth saline was essentially that of Andrewes and Harisjades(2). For preparation of mouse brain suspensions, the brains of 2 mice of an infected litter were removed aseptically, weighed, ground in a mortar, suspended in serum broth saline to make a 10% suspension, and then centrifuged lightly. The resultant suspension was then used for preparing dilutions used for inoculating mice in-

tracerebrally or rabbits intracutaneously. Rabbits were usually inoculated in the flanks with diminishing 10-fold dilutions. This served both to indicate positive transfer through the mouse brain and to determine the changes in virus titer incident to such passage.

Results. In one experiment, 4, 7, 16, and 31 days after initial infection of a litter of mice, 10% brain suspensions were prepared. Each of several sites on the flanks of a test rabbit were inoculated with 0.25-ml quantities of dilutions of this suspension, calculated to contain either 5 or 50 infectious doses. Tumors resulted at all sites on all the animals except the one inoculated with mouse brain infected for 16 days. In the second similar experiment, tumors developed on rabbits as a result of inoculation with mouse brain suspensions 3, 7, 11, and 26 days after infection but again, not as a result of the inoculation with a suspension prepared 16 days after infection. There was no apparent variation in the time of appearance or rate of growth of the tumors as a result of inoculations at different intervals from the time of mouse infection.

In several experiments involving brain-to-brain passage, transfer was positive in only one series in which 3 serial passages were accomplished. The virus dilution from mouse to mouse was approximately 250-fold, each mouse in the third transfer theoretically receiving only 2.5×10^{-5} infectious doses. The titration in a rabbit of the brains of the mice of the third passage was 10^{-4} per 0.25 ml, only a 10-fold drop from the titer of the original tumor tissue used. Unfortunately, the mouse brain passage was discontinued during the absence of the author and was unsuccessful when later resumed with some of the frozen brain material of the third passage. In view of the difference in virus yield from the brains of litter mates shown to exist in myxoma passage(2), it is probable that the

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† With the technical assistance of James C. Cunningham.

‡ Laboratory of Tropical Diseases.

§ Kindly supplied the author by Dr. Richard E. Shope of the Rockefeller Institute for Medical Research.

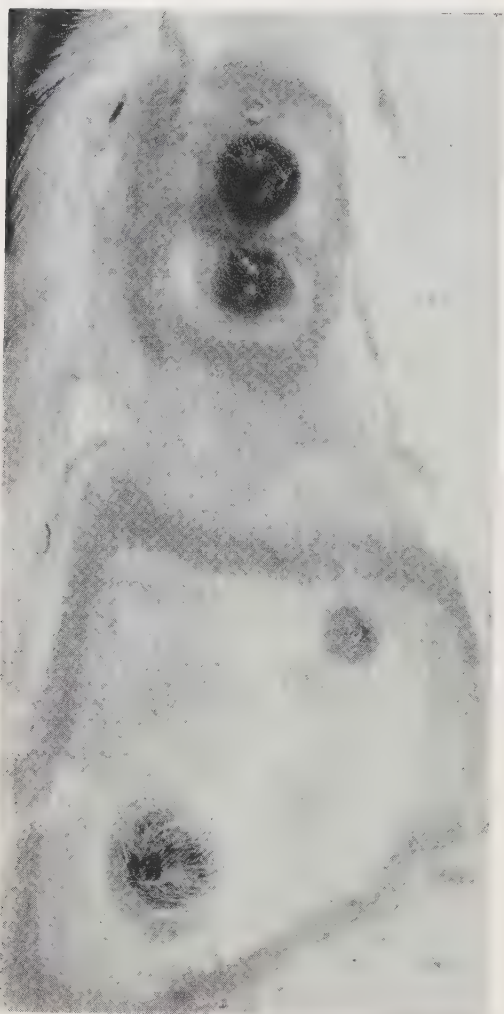


FIG. 1. Fourteen-day fibromas produced by inoculation of suspensions of mouse brains infected 7 days previously. These tumors were infective for mosquitoes when tested at 15 days.

particular tissues that had been preserved were of extremely low titer or completely negative. In all other series, the mouse-to-mouse passages were unsuccessful, although virus was always recovered from the brains of the first litter infected, as shown by inoculations into the skin of a test rabbit. Attempts to establish the virus by intracutaneous and subcutaneous inoculation into mice failed.

Mosquito (*Aedes aegypti*) [L.] transmis-

||"Infectivity" as used in this paper refers to availability of the virus in the tumors to arthropods as demonstrated by ability of the arthropods to transmit it to a second host.

sion was attempted from the 14-day-old domestic rabbit tumors (Fig. 1) resulting from inoculation of a suspension of first passage mouse brain. Normally, mosquitoes are unable to transmit virus from domestic rabbit fibromas while they can from cottontails (3,4). However, in this instance the refeeding of the mosquitoes, as well as inoculation of their mouthparts, resulted in good tumor formation. Transmission was attempted from other tumors resulting from inoculation of first and second passage mouse brain suspension, and these were also successful.

Summary. The fact that the virus titer, as shown by intracutaneous inoculations into rabbits, declined only slightly in the 3 serial passages through mice, despite a 250-fold dilution at each passage, indicates the possibility of virus proliferation. However, too few experiments were performed to establish this relationship clearly. There is no doubt that the virus can persist in the brain of the living mouse (one-day-old) for periods at least up to a month. With further attempts at mouse-to-mouse passages it appears likely that the virus can be established in this host.

While fibromas of domestic rabbits that were produced by direct inoculation of tumor suspensions are usually non-infective|| for arthropods, those tumors resulting from arthropod transmission from the natural cottontail rabbit hosts are often infective (4). Treatment of the recipient domestic rabbits with x-rays or carcinogens prior to infection with the virus also effectively changes the infectivity of the resultant tumors so that they are infective for arthropods (4,5). It now appears that passage of the virus through one-day-old mouse brain, which does not seem to affect the mouse, does alter the virus so that subsequent inoculations into domestic rabbits produce infective tumors.

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Frozen Cells as Nuclear Source in the L. E. Cell Phenomenon. (23695)

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The L.E. (lupus erythematosus) cell phenomenon(5) consists of two events: the peculiar degeneration of a cell nucleus, and the engulfment of the latter by a phagocyte. The phenomenon apparently occurs if (a) an abnormal factor, present in the gamma globulin fraction(2) of patients with systemic lupus erythematosus, is in contact with (b) cells which in some fashion have been injured, and (c) if active phagocytic cells are available to engulf the transformed nucleus of the injured cell. For reasons not yet apparent, the degenerated nucleus at times is not chemotactic and remains unphagocytosed; when unphagocytosed, this mass is referred to as L. E. material or an L. E. body. A method for producing a large mass of this unique material would allow further study of its chemotaxis (or absence of it), chemical nature, physical properties, and immunologic nature. Direct observation and histochemical analysis of fixed thin blood films have been the major means by which information regarding this unusual cellular phenomenon has been accumulated. The following method, which involves cold injury,* allows the production of large numbers of living L. E. cells and a relatively large quantity of L. E. material suspended in a liquid medium.

Materials and methods. Leukocytes from the circulating blood of human or other species served both as the donors for the altered nuclei and as the phagocytic cells. The leukocytes were separated from 5 ml of whole heparinized blood by the addition of 1.0 ml of 5% dextran† in normal saline solution. Rouleau formation allowed the erythrocytes to fall rapidly to the bottom of the tube, and the supernatant dextran and plasma, containing most of the leukocytes, was removed. Injury

to the plasma-suspended leukocytes was then produced by freezing to minus 50°C; it was possible to keep the preparation at this temperature for many months. If the plasma and leukocytes were taken directly from a patient with lupus erythematosus, they were frozen immediately. At times the source of the leukocytes and plasma was some normal human donor, or perhaps another species (*e.g.*, guinea pig), in which case serum from a lupus patient was incubated (37°C) in contact with the cells for one hour prior to freezing. Ordinarily 0.2 ml of serum from a patient with active L.E. was an adequate volume to produce the L.E. cell phenomenon when added to 1.0 ml of normal leukocyte-containing plasma. To allow the final phase of the L.E. cell phenomenon to occur, the frozen material was brought to 37°C, and fresh, active, human leukocytes were added. These were obtained from human blood by the above described dextran technic; gentle centrifugation of the leukocyte-containing plasma fraction (1200 rpm for 10 minutes) followed by decantation of most of the plasma allowed addition of many leukocytes in a small volume. These leukocytes acted phagocytically upon the nuclei which had been injured (frozen) in the presence of the L.E. factor. For microscopic examination, 1.0 ml of the preparation was gently centrifuged in a Wintrobe tube, the sediment then examined. A combination of phase contrast microscopy of an unstained wet preparation and routine Giemsa stain of fixed blood film was the usual means of observation.

Results. By this technic surprisingly great numbers of L.E. cells and free L.E. bodies are produced. If a potent L.E. factor is present, practically every frozen nucleus becomes homogeneous, purple-staining (Giemsa), Fuelgen-positive material which exhibits varying degrees of positive chemotaxis toward freshly added polymorphonuclear and mononuclear leukocytes. The stained L.E. cells (Fig. 1) resulting are in no way different from those

* The authors gratefully acknowledge comments of Dr. George Brecher, N.I.H., which led to use of freezing as means of cell destruction.

† Dextraven—Brand of dextran, produced by Bengel Laboratories Limited, Holmes Chapel, Cheshire, England.

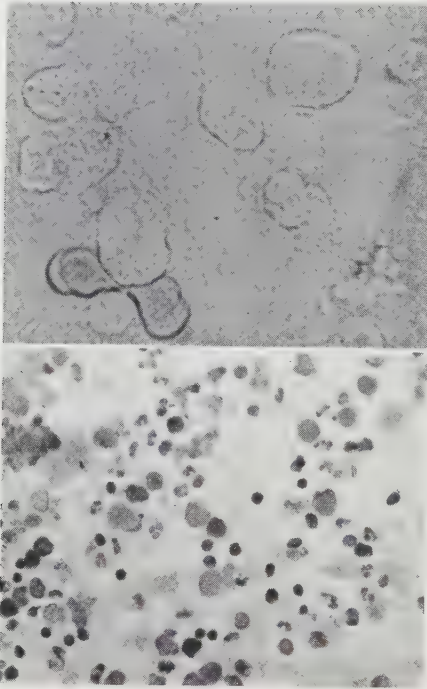


FIG. 1 (top). Two L.E. cells and several free L.E. bodies. Guinea pig cells frozen with L.E. serum. Fresh human WBC added. H & E, $\times 470$.

FIG. 2 (bottom). L.E. cell and L.E. bodies exhibiting chemotaxis. Erythrocytes for size comparison. Phase contrast, $\times 800$.

originally described by Hargraves(1) and produced in various ways by numerous authors in recent years. They exhibit the peripherally compressed, usually multi-lobate nucleus and the engulfed homogenous Fuelgen-positive mass.

Under phase contrast microscopy, observation of the chemotaxis and phagocytosis in the unstained wet preparation provides far more knowledge as to the dynamics of this phenomenon(3,6). Time-lapse micro-cinematography has proved of value in recording and clarifying these events. The L.E. bodies are observed to be globular masses of varying degrees of symmetry, often quite spherical (Fig. 2, 2a). Their margins are distinct, in contrast to the shadowy, often amorphous character of nuclei which have undergone freezing in the absence of the L.E. factor. At times small circular outlines suggestive of nucleolar remnants are seen inside the L.E. bodies. L.E. bodies tend to be free of cytoplasmic rem-

nants. These bodies usually attract phagocytes, sometimes from considerable distances, and at times more than one active leukocyte may be attracted. If several of these cells attach themselves to the L.E. material the size of the resulting mass is quite large. It is in the wet preparations that the diameter of the L.E. cell is most easily observed to be greater than normally circulating formed elements of the blood (Fig. 2).

Unexplained is the fact that some material, seemingly identical in appearance to chemotactic, Fuelgen-positive L.E. material is completely unattractive to active leukocytes in its neighborhood. In some preparations, production of L.E. cells seems to cease only when all available phagocytic cells have transformed themselves into L.E. cells. In rare preparations all of the freshly added leukocytes seem depressed, tending to become rounded, non-motile, and non-phagocytic.

Since the L.E. cell has been defined in respect to its fixed and stained characteristics (5), the living unstained preparation alone without a stained film cannot be utilized diagnostically at this time. In supravital preparations there may be various confusing cellular phenomena such as vacuolization, erythrophagocytosis, leukocytophagocytosis, nucleocytophagocytosis, or the Kurloff cell formation if fresh guinea pig cells are being observed. If a preparation is to show L.E. cells in the stained film, however, the living preparation also can be seen, by the trained observer, to contain these cells.

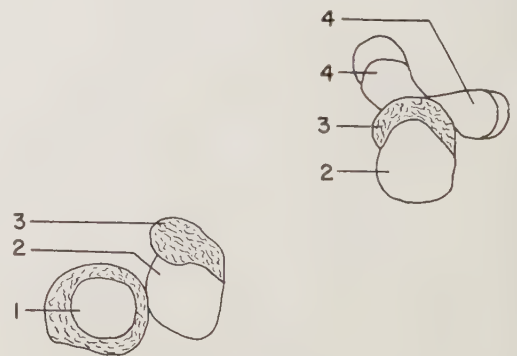


FIG. 2a. Diagram of Fig. 2.

1. L.E. cell
2. Chemotactic L.E. body
3. Phagocytic granulocyte
4. Distorted erythrocytes

TABLE I. Comparison of Bead Traumatization and Freezing Technics in Production of the L. E. Cell Phenomenon.

| Patient's own cells | Tests |
|---|-------|
| Positive using beads; positive using freezing | 11 |
| Negative " " ; negative " " | 44 |
| Positive " " ; " " " " | 4 |
| Negative " " ; positive " " | 0 |
| Patient's serum and GP cells | |
| Positive using beads; positive using freezing | 11 |
| Negative " " ; negative " " | 92 |
| Positive " " ; " " " " | 0 |
| Negative " " ; positive " " | 5 |

The widely accepted technic utilizing rotating glass beads as the source of trauma (4) to produce this phenomenon was the control standard in the present study. Blood and sera from many normal individuals and from individuals with various infectious, neoplastic, immunologic, and idiopathic diseases were subjected both to the freezing technic and to the bead traumatization technic.

The L.E. cell phenomenon could be produced using the freezing technic only when the blood of the individual also exhibited, or had exhibited, the phenomenon using the bead technic. Table I summarizes the tests on single samples of blood or serum using both technics. In 22 instances positive tests were obtained using both traumatization and freezing technics. Using heparinized patient blood, 4 instances were found in which the test was positive using beads but negative using freezing. Using stored patient serum with guinea pig cells, however, 5 instances were found in which freezing brought out the phenomenon though the bead traumatization test was negative. In these last 5 instances, the blood of the patients had at other phases of the illness been demonstrated definitely positive using beads.

Stained films of frozen preparations from which the L.E. factor is absent, show many large finely reticulated or lacy, relatively amorphous nuclear remnants which are not attractive to, nor engulfed by, phagocytes. No L.E. cells are produced.

The use of cold injury to the leukocytes in producing this phenomenon provides the advantages of (a) a large yield of L.E. material and L.E. cells, (b) freely suspended L.E. material and L.E. cells in a liquid medium without large cellular aggregates, and (c) ability to maintain the L.E. material indefinitely at minus 50°C.

Summary. 1) Cold (minus 50°C) injury applied to leukocytes in the presence of the L.E. cell factor results in production of a large amount of L.E. material. This material usually exerts chemotaxis and, in the presence of fresh phagocytes at 37°C, allows the formation of great numbers of typical L.E. cells. In the absence of the L.E. cell factor, no L.E. material nor L.E. cells are formed. 2) The large mass of this unique material which can thus be produced now lends itself to analytic methods and experimental procedures not previously applied.

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Normal Values of Swine Serum Proteins.* (23696)

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Previous reports of normal swine serum and plasma proteins have indicated a slight disparity concerning total protein concentrations and the numbers and values of constituent fractions. Heretofore these analyses utilized the moving boundary method of electrophoresis. The results of this study provide statistically reliable swine serum protein values as determined by the biuret and zone electrophoretic methods.

Methods. The blood serum samples were obtained from 79 hogs at the time of slaughter. The animals' ages varied between 5½ and 6½ months, comprising a random class regarding size and sex. The hogs were "buyers' Grade 1 and 2." They represented a geographical rearing area including the states of Nebr., Colo., and N. Mex. Total serum proteins were determined by the biuret method(1). A sample contained 0.2 ml of serum, 4.8 ml of 0.85% saline and 5 ml of biuret reagent. The samples were incubated in a 50°C, constant temperature water bath for 30 minutes. An optical density comparison with a bovine plasma albumin standard (Armour) was recorded on the Beckman B spectrophotometer at a wave length of 550 μ . Procedure for determining serum protein fractions involved the use of a horizontal strip paper electrophoresis apparatus(2). The supporting strip consisted of Whatman #1 filter paper, one inch in width. Serum was applied by two methods. In the one procedure, 0.02 ml of serum was pipetted onto the edge of a glass microscope slide and placed transversely to a predetermined section of the filter paper strip. The other method utilized filter paper, 1 mm wide and 2.2 cm long, saturated in serum and positioned on the supporting strip for the duration of electrophoresis(3). The latter procedure was preferred because it produced more consistent results

between numerous analyses and allowed greater ease in applying a uniform distribution of serum. Electrophoretic fractionations were effected in barbital buffer, (pH 8.6 μ = 0.1) after 16 hours at 120 volts and 2 ma. Upon completion of the separation, the paper strips were stained for 10 minutes in a saturated Ponceau 2R solution containing 1 part acetic acid, 4.5 parts methyl alcohol and 4.5 parts distilled water. The unbound dye was removed by successive washings in the above mixture. The strips were air dried and the stained protein fractions were cut out and eluted in 5 ml of 0.1N NaOH. The optical densities were recorded at a wave length of 465 μ . For evaluation purposes, many strips were quantitated with the Spinco Analytrol automatic recording densitometer, prior to the spectrophotometric determinations. Albumin-globulin (A/G) ratio was calculated from the electrophoretic data. Albumin was also salted out with 23% Na_2SO_4 , (Howe fractionation) and an A/G ratio obtained by this method(4) was compared with the electrophoretic measurements. The statistical computations were performed by the use of standard formulae(5).

Results. The protein patterns were well resolved into the albumin, alpha, beta and gamma globulin fractions. The constituent protein concentrations were derived from relative percentage values and are included in Table I. The numerous paper electrophoretic separations of swine serum conducted in this laboratory have failed to indicate the presence of an alpha₁ globulin which has been previously reported(6,7,9,11). The albumin-globulin (A/G) ratio as derived from the electrophoretic measurements gave a mean value of 0.88 ± 0.025 (one standard deviation). The standard error was 0.003 with 95 and 99% confidence limits of 0.005 and 0.007 respectively. Results from 20 samples of albumin determinations by Howe fractionation disclosed less than a 0.2 g% difference when

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TABLE I. Percent* and g/100 ml of Total Serum Proteins.

| | Albumin | α | β | γ | g/100 ml |
|--------------|-----------|----------|---------|----------|----------|
| | (%) | | | | |
| Mean | 46.0 | 20.0 | 14.5 | 19.5 | 7.4 |
| Stand. dev. | ± 5.8 | 4.0 | 1.6 | 4.3 | .6 |
| Stand. error | $\pm .7$ | .4 | .2 | .5 | .1 |
| t_{95} | ± 1.3 | .9 | .4 | 1.0 | .1 |
| t_{99} | ± 1.7 | 1.2 | .5 | 1.3 | .2 |
| | g/100 ml | | | | |
| Mean | 3.4 | 1.5 | 1.1 | 1.4 | 7.4 |

* Percentage values were derived from optical density measurements of each fraction, i.e. % = O.D.

Σ O.D.

t_{95} = 5% probability level; t_{99} = 1% probability level.

compared to the albumin values derived by the electrophoretic method.

Discussion. These analyses provided statistically reliable total serum protein determinations, including constituent composition values. Although there are reports of per cent protein content of swine serum(6,7,8) only Cartwright *et al.*(9) specifically indicated a mean serum total protein value of 6.70 g/100 ml, as determined from 5 animals. The Handbook of Biological Data(10) reports a mean total plasma protein value of 8.7 g/100 ml, ranging from 7.9 to 10.3.

It would be hazardous to derive valid serum protein values of individual components from plasma studies. Koenig and Hogness (6) found that fibrinogen, when isolated and subjected to electrophoresis, was contaminated with various globulin components and a small amount of albumin. Deutsch and Goodloe(11) stated that fibrinogen comprised 13.9% of the total plasma protein. If this factor is subtracted from the gram per cent of total plasma proteins(10), the resulting value is in excellent correlation with the total serum protein reported in this paper. This study also supports other reports(6,7) regarding per cent composition of serum proteins.

The fast moving component "f" of pig serum detected by Deutsch and Goodloe(11) and named rho by Williams and Grabar(12) is not discernible by this method of zone electrophoresis. However, the "f" fraction has been isolated consistently in this laboratory

using a 1% agar as the supporting medium for separation of pig serum proteins.

The existence of an α_1 globulin fraction has been noted in several reports(6,7,9,10,11), comprising from 3 to 6% of the total proteins when the isolation was performed in a veronal buffer, pH 8.6. The method of zone electrophoresis employed in this study, using a comparable buffer and pH, did not evince the α_1 fraction on the paper strip. Moore(7) utilizing a moving boundary apparatus, detected an α_1 with a barbiturate buffer pH 8.6, but failed to do so in a phosphate buffer of pH 7.4. Svensson's(8) moving boundary patterns also failed to disclose an α_1 globulin. The buffer used was phosphate, pH 7.7, which may account for the absence of this fraction as shown by Moore(7).

Evaluation of the data from this study, reflected upon the serum and plasma values of previous investigations(6,7,8,11) substantiates the probability of the α_1 fraction migrating with albumin. It is evident that salted out albumin contains the α_1 globulin, as shown by the resulting concentration being in similitude with albumin derived electrophoretically when α_1 has not been resolved as a separate fraction or by adding the α_1 value, when separated, to the albumin factor.

Summary. 1) Zone electrophoretic examination of sera from a large, random selection or apparently healthy hogs has yielded statistically valid protein concentrations. 2) Under the conditions of this experiment, only albumin, alpha, beta, and gamma globulin were resolved and quantitated. 3) The "f" fraction (11) and α_1 globulin are discernible only under specific conditions. The ionic strength, the pH of the buffer and apparently the separating medium influence the resolution of the "f" and α_1 fractions.

Appreciation is expressed to Schwartzman Packing Co. of Albuquerque, N. Mex. for supplying the pig sera samples.

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Effect of Sex Hormones on Ethanol Induced Fatty Infiltration of Liver in Rats. (23697)

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It has been previously reported that either chronic or acute ethanol intoxication causes lipid to accumulate in the liver of rats(1,2), and that under the same conditions of acute ethanol intoxication, female rats show a more severe fatty infiltration of the liver than do males(2). The experiments reported below were therefore carried out in order to determine the effect of male and female sex hormones on alcohol-induced fatty infiltration of the liver.

Procedure. Eight groups of male and 6 groups of female albino rats, weighing 70-80 g at the start, were maintained on Purina dog chow pellets *ad libitum* for periods of either 4 or 6 weeks. Three of the groups of males and 2 of the groups of females were castrated at the beginning of the experiment. During these periods of time, two groups of intact males received daily intramuscular injections (0.1 ml) of estradiol benzoate in sesame oil which was diluted with Wesson oil, and one such group received daily injections of 0.1 ml of Wesson oil alone. The dose of estradiol benzoate was 0.005 mg/day during the first 2 weeks, and 0.01 mg/day during the last 4 weeks. Testosterone propionate in oil (0.1 ml) was similarly administered to 1 group of castrated males, the dose being 0.1 mg/day during the first 2 weeks and 0.2 mg/day during the last 2 weeks, and to 2 groups of intact females, the dose being 0.1 mg/day during

the first week, and increased weekly to 0.4 mg/day. The remaining groups received no injections. All animals weighed approximately 200 g at the end of the 4- or 6-week periods, when they were killed by decapitation. The longer period was required only for those rats receiving estrogen, because the hormone, in the dosage given, was found to exert a small inhibitory effect on the rate of gain of weight. At the end of the 4 or 6 weeks, the rats in 8 of the groups received a single intoxicating dose of ethanol (4.0 ml of a 50% solution by volume) by stomach tube, while those in the remaining groups received a single dose of glucose, calorically equivalent to the dose of alcohol, in the same volume of solution, and in the same manner. All food was then withdrawn, the female rats killed 18 hrs and the male rats 16 hrs thereafter. The livers were immediately removed, samples of liver frozen and subsequently analyzed for total lipids as already described(2). The 18- and 16-hour intervals subsequent to stomach tubing were chosen because it was previously found that female and male rats show peak depositions of liver lipid, subsequent to ethanol intoxication, at these times(2). The seminal vesicles were also removed from 6, and testes from 3 of the groups of males at the time of killing. The fluid was expressed from the seminal vesicles, and these organs as well as the testes weighed on a Roller Smith balance.

TABLE I. Effect of Sex Hormones on Fatty Infiltration of Liver of Ethanol Intoxicated Rats.

| Group | No. rats | Ethanol or glucose | Hormone or oil (daily dose) | Total liver lipid, % | P | Sem. vesicles, mg/100 g body wt | P | Testes, mg/100 g body wt | P |
|----------------------------|----------|--------------------|---|----------------------|---------------------------------------|---------------------------------|-----------------|--------------------------|-----------------|
| Intact male rats | | | | | | | | | |
| 1 | 11 | Glucose | | 5.5 ± .11 | | 116.3 ± 5.97 | | 1173 ± 53.7 | |
| 2 | 11 | " | .005-.01 mg estradiol benzoate in oil | 5.8 ± .17 | P > .1 (1 & 2)* | 60.4 ± 3.69 | P < .01 (1 & 5) | 173.4 ± 5.53 | P < .01 (1 & 5) |
| 3 | 13 | Ethanol | | 7.7 ± .16 | P < .01 (1 & 3) | | | | |
| 4 | 8 | " | .1 ml in oil | 7.6 ± .33 | P > .1 (3 & 4) | | | | |
| 5 | 9 | " | .005-.01 mg estradiol benzoate in oil | 7.7 ± .26 | P > .1 (3 & 5) | 56.9 ± 3.16 | P < .01 (1 & 5) | 180.1 ± 15.4 | P < .01 (1 & 5) |
| Castrated male rats | | | | | | | | | |
| 6 | 11 | Glucose | | 6.2 ± .17 | P < .01 (1 & 6) | 9.2 ± .37 (30 rats) | P < .01 (1 & 6) | | |
| 7 | 19 | Ethanol | | 9.7 ± .37 | P < .01 (3 & 7) | | | | |
| 8 | 10 | " | .1-.2 mg testosterone propionate in oil | 6.7 ± .18 | P < .01 (3 & 8) | 220.8 ± 13.9 | P < .01 (1 & 8) | | |
| Intact female rats | | | | | | | | | |
| 9 | 10 | Glucose | | 6.7 ± .21 | P < .01 (9 & 11) | | | | |
| 10 | 12 | " | .1-.4 mg testosterone propionate in oil | 5.8 ± .12 | P < .01 (9 & 10) | | | | |
| 11 | 11 | Ethanol | | 10.7 ± .50 | P < .01 (11 & 12) | | | | |
| 12 | 12 | " | <i>Idem</i> | 7.5 ± .13 | P > .1 (12 & 13) | | | | |
| Ovariectomized female rats | | | | | | | | | |
| 13 | 8 | Glucose | | 5.6 ± .09 | P < .01 (9 & 13) P < .01 (13 & 14) | | | | |
| 14 | 24 | Ethanol | | 10.2 ± .29 | P > .1 (11 & 14) | | | | |

* Numbers in parentheses refer to groups.

Results. (A) *Alcohol-induced fatty infiltration of the liver.* The experimental data are summarized in Table I. Prior administration of estradiol to intact male rats did not alter the ethanol-induced fatty infiltration of the liver (Group 3 *vs.* 5) although the weights of seminal vesicles and testes indicated that a potent dose of estrogen had been administered (Group 1 *vs.* 2 and 5). There was also no effect of ovariectomy of female rats on liver lipid accumulation (Group 11 *vs.* 14).

In contrast to this lack of effect of estrogen, androgen exerted a marked influence on the

amount of liver lipid present after ethanol administration. Thus, castrated male rats (Group 7) accumulated much more lipid than did intact males (Groups 3 and 4). In fact, castrated males showed as high a liver lipid concentration as did females, there being no statistically significant difference between the 2 groups (Group 7 *vs.* 11). When castrated males received testosterone however (Group 8), this increase in liver lipid concentration over that in intact males, disappeared. Indeed, even less lipid accumulated in the livers of the testosterone treated, castrated male rats

than in the livers of intact males (Groups 3 and 8). This was probably due to the administration of excessively large quantities of testosterone, as indicated by the significantly greater weights of the seminal vesicles of castrated males receiving testosterone, than of intact males receiving no hormone (Group 8 *vs.* 1). In accordance with the above observations on male rats, it was found that intact female rats receiving testosterone showed a significant decrease in ethanol-induced accumulation of liver lipid. Thus, these animals showed the same liver lipid concentrations as did intact males, in response to ethanol administration (Groups 12 and 3).

Injection of oil vehicle itself did not influence the level of liver lipid (Group 4 *vs.* 3).

(B) *Liver lipid in non-alcoholic rats.* Since it was thought that castration, or the administration of hormone, might affect liver lipid values, apart from ethanol intoxication, controls were run in which isocaloric quantities of glucose were administered in place of ethanol, to operated and hormone treated rats. These procedures did, in some instances, alter the normal liver lipid concentrations, but only to a small degree, and not enough to mask the larger effects on the fatty infiltration of the liver promoted by ethanol intoxication. Thus the administration of testosterone to intact females receiving glucose produced a small but significant decrease in liver lipid concentration (Group 10 *vs.* 9). Castrated male rats receiving glucose, on the other hand, had small but significantly higher liver lipid values than did glucose-fed intact rats (Group 6 *vs.* 1). Thus the effect of male sex hormones on the control liver lipid values was qualitatively the same as that on the liver lipid values following ethanol intoxication, but quantitatively much smaller.

Administration of estrogen to males exerted no influence on the liver values of the glucose controls (Group 2 *vs.* 1). This too was similar to the lack of effect on liver lipids after ethanol intoxication. On the other hand, ovariectomy of female rats did produce a small but significant decrease in liver lipid concentration of the controls (Group 13 *vs.* 9).

Discussion. It may be considered possible

that the apparent lack of effect of estradiol on the ethanol-induced fatty livers was in reality a resultant of two opposed forces: (1) a direct fatty liver promoting effect and (2) an indirect inhibitory effect (*via* the pituitary) on fatty liver production. Thus estradiol did have some inhibitory effect on rate of growth as well as on testicular and seminal vesicular weight (Group 1 *vs.* 2 or 5), presumably largely *via* pituitary inhibition, and it has been previously observed that hypophysectomy prevents ethanol-induced fatty infiltration of liver in rats(2). However, since animals receiving estrogen still had substantially larger quantities of testosterone available than did castrated rats, as seen from seminal vesicular weights (Groups 2 and 5 *vs.* 6); since administration of estradiol produced no change whatsoever in liver lipid concentration (Group 3 *vs.* 5); and since ovariectomy of female rats also produced no change in liver lipid after alcohol, the first and simpler explanation appears preferable.

Reports have appeared in the literature that procedures other than ethanol administration also result in a differential fatty infiltration of the liver between the sexes. Thus, more lipid has been found to accrue in the livers of female than of male rats after partial hepatectomy(3), the administration of diets low in protein and high in fat(4,5), and the administration of pituitary extracts(6). A lipotropic activity of male sex hormones would help to explain these phenomena also.

György and co-workers found, however, that in the case of fatty livers produced in rats by diets low in protein and high in fat, testosterone exerted no lipotropic effect, while the estrogens did exert such an effect(7,8). It was noted that the lipotropic action of estradiol benzoate was manifested only when methionine was also administered. On this same diet, female rats showed a greater increase in liver fat after ovariectomy, but castration had no effect on the fatty liver of male rats(9). These results are in contrast to our observations on ethanol-induced fatty livers. It is therefore possible that the particular effect of the sex hormones on liver lipid may vary with the mechanism that produces the fatty infiltration.

Summary. Female rats showed a more severe fatty infiltration of the liver than did male rats following ethanol intoxication. Administration of estradiol to male rats, or ovariectomy of female rats, prior to such intoxication, did not affect this fatty infiltration. On the other hand, administration of testosterone to female rats reduced the liver lipid concentrations to values similar to those obtained with males, while castration of males increased the liver lipid concentrations to those obtained with females. Castrated male rats, when treated with testosterone however, no longer showed liver lipid values that were higher than those of intact males. These results suggest that testosterone exerts a lipotropic action on ethanol-induced fatty infil-

tration of the livers in rats, while the estrogens do not influence this process.

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Diurnal Variation in Mouse and Rat Liver Sulfhydryl.* (23698)

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In the course of experiments performed for another purpose it was noted that otherwise untreated mice sacrificed in the afternoon exhibited significantly smaller liver non-protein sulfhydryl concentrations (NPSH) than did untreated mice sacrificed in the morning of the same day. This paper is concerned with an investigation of this phenomenon.

Methods. Liver extracts were secured by grinding the tissue with ice-cold 2% sulfosalicylic acid or 6% HPO_3 , 19.3 ml/g, to give a 20-fold dilution of tissue NPSH. Filtered extracts were held in a refrigerator to time of analysis, always on the same day. The nitroprusside method of Grunert and Phillips(1) for estimation of NPSH was modified to assure adequate neutralization of HPO_3 , to minimize effects of fading of the nitroprusside color, and to fit our equipment (Bausch and Lomb spectrophotometer, 1 inch cuvettes,

wave length 525 $\text{m}\mu$). The procedure employed was as follows: (a) for the sulfosalicylic acid extracts and solutions, 2.5 ml of the Grunert-Phillips carbonate-cyanide reagent and 2.5 ml of 2% sodium nitroprusside were placed in a large test tube; for the HPO_3 extracts and solutions, 3 ml of the carbonate-cyanide reagent and 2 ml of 2.5% Na nitroprusside were placed in a large test tube, (b) 2 ml of the NPSH containing extract or solution and 18 ml of saturated NaCl were placed in a cuvette, (c) with the cuvette in place, the spectrophotometer was adjusted to read ZERO optical density, (d) the contents of the test tube and cuvette were rapidly and thoroughly mixed and the test optical density reading obtained. In certain of the experiments (Table I), not only was NPSH of each extract estimated, but also glutathione (GSH) by the alloxan method of Lazarow(2). This method is one which Lazarow has stated is not entirely satisfactory for tissue extracts, although suitable for blood. Sulfosalicylic acid liver extracts could not be employed be-

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TABLE I. Further Studies on Relation between Time of Day and Liver Sulfhydryl.

| Time of day | Liver NPSH and GSH as GSH equiv., mg %, \pm stand. error* | | | | | | | |
|-------------|---|------|----------------|----------------|--|--------------|---|--------------|
| | Untreated rats (Exp. 3)† | | Untreated mice | | Sham operated mice (Exp. 6 + Exp. 7)§ | | Adrenalectomized mice (Exp. 6 + Exp. 7)§ | |
| | NPSH | GSH‡ | NPSH Exp. 4 | NPSH Exp. 5 | NPSH | GSH | NPSH | GSH |
| 8 A.M. | 260 \pm 6 | 208 | 284 \pm 6 | 304 \pm 8 | 352 \pm 14 | 290 \pm 12 | 259 \pm 13 | 209 \pm 15 |
| 12 noon | 226 \pm 7 | 199 | 303 \pm 8 | 332 \pm 12 | 274 \pm 13 | 229 \pm 9 | 280 \pm 15 | 227 \pm 9 |
| 4 P.M. | 200 \pm 7 | 184 | 219 \pm 10 | 230 \pm 12 | 268 \pm 12 | 209 \pm 8 | 248 \pm 12 | 207 \pm 12 |
| 8 " | 179 \pm 6 | 113 | 219 \pm 11 | 201 \pm 9 | 227 \pm 13 | 170 \pm 12 | 207 \pm 8 | 146 \pm 12 |
| 12 midnight | 182 \pm 7 | 160 | 224 \pm 9 | 230 \pm 8 | 261 \pm 10 | 230 \pm 9 | 251 \pm 11 | 209 \pm 11 |
| 4 A.M. | 234 \pm 7 | 211 | 283 \pm 7 | 274 \pm 9 | | | | |

NPSH = non-protein sulfhydryl; GSH = glutathione.

* Each mean value is for analyses on 9 to 12 separate livers.

† Rat wts, 140-180 g.

‡ One

analysis only, on a pooled extract.

§ Data for Exp. 6 and 7 pooled.

cause of strong absorption at 305 $m\mu$. Absorption by 6% HPO_3 was small. However, appreciable absorption at 305 $m\mu$ did occur for 6% HPO_3 liver extracts not containing alloxan. Hence the GSH values of Table I must be considered as being less reliable than the corresponding NPSH values. The mixture employed for securing a test optical density reading for a given extract was prepared as follows: (a) the original tissue extract was diluted 4, 5 or 10 times with 6% HPO_3 , (b) 2 ml of this *diluted* extract were mixed with 3 ml of M/3 sodium phosphate buffer of pH 7.55, 2 ml of 0.1 N alloxan and 1 ml of 1.25 N NaOH (pH of this mixture about 7.45), and (c) 7 minutes later, 2 ml of 1.0 N NaOH were added and the test optical density at 305 $m\mu$ secured immediately, using a Beckman DU spectrophotometer. The *blank* for the same extract was prepared in the same manner except that all alkali solutions were added before the alloxan, in order to prevent formation of the alloxan-glutathione complex, and the 7 minute waiting period was eliminated. CFW male mice, 20-26 g body weight, were employed. In Exp. 4 and 5 (Table I) the mice were held in an air-conditioned room at 25-27°C during and for 4 days before sacrifice. Wistar strain female rats were used, of body weights indicated under Results. Operations (sham or adrenalectomy) were performed on mice 4 days before the day of sacrifice. Due to the time required for these operations the sum total of the data of Exp. 6 and 7 (Table I) could be obtained only by performing 2 separate experiments, with resulting greater scatter of pooled data than

typically occurs in a single experiment. The animal housing rooms had daylight exposure. Rats were held in open mesh wire cages, mice in transparent plastic cages with sawdust on the bottom. Food was available at all times and consisted of Rockland rat or mouse pellets. Food and water were replenished in the rat cages each morning, in the mouse cages when necessary. Animals were placed in clean cages a day or so before any given experiment. All of the animals to be sacrificed at a given time were placed in the same cage or set of cages, to minimize disturbance of animals not scheduled to be sacrificed at that time.

Results. Two experiments were performed in which control (untreated) animals were sacrificed at 1½ hour intervals over the period between about 9:30 a.m. and 4:30 p.m. (the maximum time spread over which control animals had been sacrificed in previous experiments). Male mice exhibited a progressive decrease in liver NPSH (expressed as mg %, GSH equivalent, \pm standard error) from a high value of 296 \pm 11 at 9:30 a.m. to a low value of 187 \pm 14 at 4:30 p.m. ($p < 0.001$). Female rats of weights 83 to 146 g exhibited a progressive decrease from a high value of 211 \pm 8 at 9:30 a.m. to a low value of 134 \pm 10 at 4:30 p.m. ($p < 0.001$). It would appear very important, therefore, that any experiment performed to test for the effect of a procedure on mouse or rat liver NPSH or GSH should be performed in such a manner that control and test animals are sacrificed at as nearly the same time of day as possible. The data of Table I are for experiments in which

animals were sacrificed at 4-hour intervals over a 20- or 24-hour period. In these experiments maximal values for both liver NPSH and GSH occurred at 8 a.m. or 12 noon, minimal values at 8 p.m. The diurnal variation in GSH was sufficient to account for all of the diurnal variations in NPSH. These relations held for untreated, sham operated and adrenalectomized male mice, whether kept in an air-conditioned room or not; they also held for untreated female rats. Diurnal variations in liver NPSH and GSH were appreciably less for adrenalectomized than for either untreated or sham operated mice.

The unexpected finding of a considerable diurnal variation in liver NPSH and GSH of untreated mice and rats made it desirable to reexamine previously reported findings(3,4) that marked decreases in liver NPSH are induced by severe trauma or exposure to cold. An experiment was performed in which 9 test mice were subjected to tourniquet trauma(5) (both hind legs ligated 2 hours, sacrifice 2 hours after removal of ligatures). Five of the untreated control mice were sacrificed immediately before and 4 immediately after sacrifice of the traumatized mice. The following liver sulfhydryl values, expressed as mg %, GSH equivalent, \pm standard error, were obtained: Control mice — NPSH = 261 ± 9 , GSH = 222 ± 7 ; traumatized mice — NPSH = 142 ± 5 , GSH = 135 ± 4 . In this experiment very much lower liver sulfhydryl values were found for traumatized than for non-traumatized mice, although the time of day at which sacrifice occurred was as nearly as possible the same for both groups of mice.

Discussion. From the literature(2) it would appear that synthesis of GSH occurs mainly in the liver. We have found(6) that dietary sulfur amino acid deficiency results in a far greater decrease in mouse liver NPSH

than in the NPSH of any of the other tissues analyzed (kidney, spleen, heart, skeletal muscle). This indicates that the liver not only manufactures but also stores GSH until it is needed elsewhere. Diurnal variation in GSH storage in the liver might be due primarily to variation in (a) dietary intake of sulfur amino acids, or (b) passage of GSH from the liver. It is well known that mice and rats exhibit appreciably greater food intake during the night than during the day; such a dietary pattern might very well result in a gradual increase during the night in the amount of GSH stored in the liver, and a gradual dissipation of stored liver GSH during the day. Whatever the mechanism, the diurnal variation in mouse and rat liver NPSH and GSH is of such a magnitude as to arouse suspicion that some reports in the literature of experimentally induced changes in mouse or rat liver NPSH or GSH may actually reflect in large part diurnal variation in GSH.

Summary. Untreated, sham operated and adrenalectomized male mice and Wistar Strain female rats all exhibited statistically significant diurnal variations in liver NPSH and GSH. High NPSH and GSH values came at 8 a.m. or 12 noon, low at 8 p.m.

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Nitrogen Content of Gastrointestinal Tracts of Rats During Absorptive Period.* (23699)

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In a series of earlier papers(1a,b,2,3) we reported the effects of stomach emptying, intestinal propulsion, and enzymatic breakdown on the absorption and utilization of dietary proteins. In these experiments adult male rats were trained to eat definite quantities of different proteins or other test meals and the nitrogen present in the gastrointestinal contents was determined at intervals following ingestion. It was found that, 2 hours after feeding, in most cases 50-70% of the ingested nitrogen disappeared from the gastrointestinal tract and the speed of disappearance and the distribution of the residual nitrogen between stomach and intestine depended upon the nature of the protein.

Conflicting results were obtained by Dreisbach and Nasset(4) who, using a similar method, consistently recovered more nitrogen from the digestive tract in rats during the absorptive period than had been fed. From these results and from experiments with dogs(5), they postulated existence of a "homeostatic mechanism" whereby the digestive tract could supply to a deficient meal the missing amino acids or those present in insufficient quantities. This is presumably accomplished by secretion of digestive juices and by desquamation of mucosal epithelium. Due to the importance of this concept, if substantiated, and due to the contradiction existing between the results of Nasset and Dreisbach and our own, we decided to investigate the problem by: (1) determining the amount of nitrogen present in gastrointestinal contents of rats after a protein-free meal; (2) studying changes in these values under conditions of progressive protein depletion; and (3) investigating the nitrogen content of the gastrointestinal tract

after feeding different skim milk powders. Skim milk powders were chosen as test meals because we had found (1) that milk protein is eliminated from the gastrointestinal tracts of rats slower than other proteins, and consequently any accumulation of endogenously supplied nitrogen would be made more conspicuous.

Methods. Male, albino rats of the Wistar strain, weighing between 140 and 220 g were kept in individual cages, water being provided at all times. Body weights were recorded daily. The animals were starved 24 hours (previous work had shown that the residual nitrogen in the gut after 24 hours was negligible) and then offered a one-half gram test meal consisting of 80% starch and 20% butterfat. Only those rats who ate the meal immediately without spilling were used. An hour and a half or 3 hours *post cibum*, rats were killed by a blow to the head. The abdominal cavity was opened and ligatures placed at the cardia and at the pyloric and ileocaecal sphincters. The intact stomach and small intestine were removed, and contents collected separately by washing repeatedly into a flask. The washing has to be performed carefully immediately after killing in order to prevent post mortem desquamation which may alter results. The washings were acidified and analyzed for nitrogen by the micro-Kjeldahl method. In several cases the accuracy of this method was controlled by Nesslerization of parallel samples and determination of nitrogen with a Lumetron colorimeter. Rats on stock diet received Rockland rat cubes. For protein depletion the rats received the following diet *ad libitum*: Cornstarch, 81.0%; butterfat, 2.5%; wheat germ oil, 2.5%; lard, 3.2%; choline HCl, 0.4%; methylolinoleate, 0.4%; U.S.P. Salt Mixture #5, 4.0%; Ruffex (methyl cellulose), 6.0%; vitamins were supplemented to previously established levels(6). Four control animals,

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TABLE I. mg of Nitrogen Present in Gastric and Intestinal Contents 90 Min. after Ingestion of a Protein-Free Meal. (Avg values obtained with 4 rats each.)

| Days on protein-free diet | mg N present 90 min. <i>post cibum</i> | | |
|---------------------------|--|-----------------|----------------|
| | In stomach | In intestine | Total |
| 0 (controls) | 1.18 \pm .41 | 4.02 \pm .57 | 5.20 \pm .80 |
| 2 | 1.75 \pm .59 | 5.09 \pm 1.00 | 6.85 \pm .42 |
| 4 | .54 \pm .17 | 5.34 \pm .96 | 5.88 \pm .91 |
| 6 | 1.31 \pm .39 | 4.01 \pm .52 | 5.34 \pm .95 |
| 8 | 1.05 \pm .38 | 2.91 \pm .27 | 3.96 \pm .60 |
| 18 | 2.01 \pm .39 | 3.08 \pm .43 | 5.09 \pm .59 |

previously fed stock diet, were starved for 24 hours and killed. Groups of 4 animals each were sacrificed after 2, 4, 6, and 8 days of protein depletion. In a second group, 2 g of various skim milk preparations, supplying 96-107 mg of nitrogen, were fed as a test meal to rats kept on stock diet. The milk powders were wetted immediately before feeding by addition of approximately 0.5 ml of water and one or two drops of tuna juice for flavor.

Results. The results of the first group of experiments are summarized in Table I. The nitrogen content of the stomachs and intestines of the control animals kept on a stock diet, fasted for 24 hours and then given the protein-free test meal, was found to be 5.20 mg (4.59-5.99 mg). Subjecting the animals to continued protein inanition did not cause any significant diminution of these nitrogen values. After 18 days on the protein-free diet, the nitrogen content was found to be 5.09 mg (3.83-6.26 mg) and on no single day was there any significant deviation from these mean values.

In the second group, skim milk powders served as test meals. The results of these experiments are condensed in Table II. The figures indicate that the amount of nitrogen recovered was in no instance higher, but always lower, than the amount of nitrogen consumed with the test meal.

The figures show also that the freeze-dried sample was digested and absorbed approximately at the same rate as the "instant" sample which was spray dried at a considerably higher temperature.

Discussion. Following a protein-free meal, the amount of nitrogen supplied by gastrointestinal secretion and desquamation was about 5 mg, *i.e.* much lower than indicated by Dreisbach and Nasset who reported an average value of 0.29 g of protein, or the equivalent of 45 mg of nitrogen. It is of interest to note here that Junqueira, Rothschild, and Fajer(7) have shown that the total *protein* production of the stimulated rat pancreas is 2.14 mg per hour. The quantities of amino acids supplied endogenously during the absorptive period seem to be far too low to make up for a dietary amino acid deficiency, at least in the rat. Protein depletion induced by feeding a protein-free diet up to 18 days does not influence the quantity of endogenous nitrogen supplied to the gastrointestinal tract. Confirming the results of our earlier experiments, we found consistently less nitrogen present during the absorptive period than had been fed. Differences in animal material and in specific experimental conditions could possibly be responsible for the differences between our results and those of Dreisbach and Nasset.

Summary. 1) The basal level of endogenously supplied nitrogen in adult rats is about 5 mg after 1½ hours, and this level is unaffected by protein depletion. On feeding skim milk powder, 22-27% of the ingested protein disappeared from the gastrointestinal tract within 3 hours. Under the conditions of these experiments the alleged homeostatic action of the gut in supplementing amino acids to deficient protein meals may be only negligible. 2) No significant differences have

TABLE II. mg of Nitrogen Present in Gastric and Intestinal Contents 3 Hr after Consumption of a Skim Milk Powder.* (Avg values for 4 animals.)

| Type of skim milk powder | mg N present 3 hr <i>post cibum</i> | | | % less than consumed |
|--------------------------|-------------------------------------|-----------------|-----------------|----------------------|
| | In stomach | In intestine | Total | |
| "Instant" (98.4 mg N) | 67.5 \pm .96 | 8.9 \pm .85 | 76.4 \pm 1.47 | 22 |
| "Low temp." (98.4 mg N) | 59.8 \pm 3.64 | 14.7 \pm 6.53 | 74.5 \pm 3.18 | 23 |
| Lyophilized (107 mg N) | 63 \pm 10.03 | 7.1 \pm .75 | 70.1 \pm 6.62 | 33 |

* Samples were supplied by Research Department of Carnation Co. of Los Angeles.

been observed in gastric emptying time, rate of absorption, or digestion between skim milk powders dried at low or high temperature.

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Different Fractions of Serum Proteins in Normal and Scorbatic Guinea Pigs. (23700)

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Electrophoretic patterns of plasma and serum proteins of a few animals have been studied(1,2) by the moving boundary electrophoresis. Greenberg *et al.*(3) observed no significant alteration in the serum proteins patterns of monkeys during early stages of scurvy. An increase in the plasma fibrinogen content has been reported in scorbatic monkeys(4) and guinea pigs(6). The present communication deals with studies on the different fractions of serum proteins in normal and scorbatic guinea pigs by paper electrophoresis as similar studies have not been previously reported.

Methods. The selection of guinea pigs and the feeding of the scorbatic diet by the paired feeding technic were the same as described previously(6). On the 30th day of the experiment when the experimental animals developed severe scurvy, food was withdrawn from the cages in the evening and on the next morning both the scorbatic animal and its normal mate were anesthetized with pentobarbital sodium and blood samples withdrawn by cardiac puncture. A small quantity of blood was taken in oxalated tube and the rest allowed to clot for the separation of serum. Total nitrogen and non-protein nitrogen of serum and plasma fibrinogen were determined by methods described previously(7). Serum proteins were separated by paper electrophoresis. 0.02 cc undiluted serum was used

and electrophoresis continued for 16 hours with a current of 1.5 mA and 100 volts. The staining of the paper strips, determination of the optimum density curves of the coloured zones by densitometry and calculation of the amounts of different fractions of serum proteins were the same as described previously (7).

Results. The results are given in Tables I and II. Six clear components of serum proteins were present in guinea pig serum. Serum albumin, α_2 and β_2 -globulin values decreased considerably and significantly, α_1 -globulins increased significantly and there was no change in γ -globulin and β_1 -globulin fractions when the guinea pigs developed scurvy. Total protein value of serum decreased, non-protein nitrogen value of serum and fibrinogen value of plasma increased in scorbatic animals.

Discussion. The lowered albumin globulin ratio in scorbatic guinea pigs agrees with similar observations reported by others(8). The decreased total plasma protein contents has also been reported in scorbatic monkeys (4). Moore(2) could not observe β and γ globulins in guinea pig sera by moving boundary electrophoresis. The demonstration of these components by paper electrophoresis indicates the superiority of the method over moving boundary electrophoresis. Fukuda and Shibatani(9) and Fukuda(10) observed

TABLE I. Different Fractions of Serum Proteins in Guinea Pigs (%).

| Guinea pigs | Albumin | Globulins | | | | |
|----------------|-----------------|---------------|----------------|---------------|---------------|---------------|
| | | α_1 | α_2 | β_1 | β_2 | γ |
| Normal (10) | 2.82 \pm .05* | .42 \pm .03 | 1.38 \pm .08 | .35 \pm .01 | .56 \pm .03 | .68 \pm .05 |
| Scorbutic (8) | 1.90 \pm .08 | .77 \pm .03 | 1.19 \pm .06 | .31 \pm .03 | .43 \pm .02 | .69 \pm .04 |
| Diff. of means | .92 | .35 | .19 | .04 | .13 | .01 |
| S.E. of diff. | .093 | .04 | .095 | .032 | .033 | .052 |
| <i>t</i> | 9.86 | 8.83 | 2.00 | 1.25 | 3.93 | .19 |

* Mean \pm S.E.

Figures in parentheses indicate No. of animals.

a decrease in liver protein values in scorbutic guinea pigs. The decrease in the serum albumin and total serum proteins values in scurvy might be due to diminished synthesis of these components by the liver of the scorbutic animal.

Elevation of fibrinogen in scurvy might be due to dysfunction of the liver. Antibodies are mainly contained in the γ -globulin of plasma proteins(11). γ -globulin values, however, did not change in scorbutic guinea pigs although it has been stated that Vit. C-nutrition influences the development of immunity in the body(12). A significant increase in the non-protein nitrogen values of serum in scorbutic guinea pigs might be due either to increased tissue catabolism or to its retention by the dysfunction of the kidney. The changes in the different components of plasma proteins in scurvy indicate the inter-relations of Vit. C-nutrition and protein metabolism in the body.

Summary. Different fractions of plasma

TABLE II. Total Serum Protein, Serum Non-protein Nitrogen and Plasma Fibrinogen Values in Guinea Pigs (%).

| Guinea pig | Serum protein | Serum N.P.N. | Plasma fibrinogen |
|----------------|----------------|---------------|-------------------|
| Normal (10) | 6.2 \pm .10 | .06 \pm .00 | .31 \pm .04 |
| Scorbutic (8) | 5.29 \pm .17 | .10 \pm .01 | .56 \pm .07 |
| Diff. of means | .92 | .04 | .25 |
| S.E. of diff. | .196 | .012 | .077 |
| <i>t</i> | 4.6 | 3.3 | 3.2 |

proteins were determined by paper electrophoresis in scorbutic and paired-fed normal guinea pigs. Guinea pig serum contained albumin and α_1 , α_2 , β_1 , β_2 and γ -fractions of globulins. There was a significant decrease in albumin, α_2 and β_2 -globulins; considerable increase in α_1 -globulin and no significant change in β_1 and γ -globulins in the serum of scorbutic guinea pigs.

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Gas Chromatographic Analysis of Alcohol and Certain Other Volatiles in Biological Material for Forensic Purposes.* (23701)

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Gas-liquid partition chromatography affords a general method of analysis for microgram concentrations of volatile mixtures that is expedient in procedure, specific, and quantitative in character. These attributes are lacking in the customary technics utilized for toxicological analysis of volatiles(1).

The previously reported use of glycol columns(2,3) have indicated their usefulness for analysis of water-alcohol mixtures. This report is concerned with the use of glycol columns for quantitative micro determinations of some volatile compounds from biological distillates which are of toxicological interest.

Method. Distillation. One to 5 cc of whole blood (urine or tissue) were pipetted into a 250 cc electrically heated distilling flask containing 25 cc of water, 3 cc of 50% sodium tungstate, and 5 cc of 1 N sulfuric acid. The vapour was passed through a heated fractionating column (Eck & Krebs #3470) for one hour. The column was 55 cm in length, 1 cm in diameter filled with glass helices. Twenty to 25 cc of distillate were collected in an ice-cooled receiver. **Gas Chromatography:** The gas chromatography apparatus used was a Perkin-Elmer Fractometer model 154B set at full Sensitivity. The recorder was a Leeds and Northrup Speedomax with 5 mv. full scale sensitivity and a chart speed of 12 in. per hour. A 0.1 cc or 0.05 cc aliquot of the distillate was injected into one of 2 glycol columns using a one-quarter cc tuberculin syringe. Column #1 was a 50:30:20 by weight, 30-60 mesh Johns-Manville C22 firebrick, glycerol, tricresyl phosphate (TCP) mixture(3) in a 1/4" copper tube 1 meter long. Column #2 was a 60:18:22 firebrick, glycerol, TCP mixture in a 1/4" copper tube 1 meter long. The identity of ethanol and other separated components was determined by adding the suspected substance to the distillate and

re-running the sample to see if the peak height in question was increased. Separated components were quantitatively assayed by direct calibration of the column.

Results. Fig. 1 shows the direct calibration for ethanol. Blood alcohols were determined to an accuracy of 5 mg % as illustrated in Table I. The concentrations of the "control" blood alcohol solutions never varied more than 5 mg % from a modified Widmark alcohol analysis(4). One microgram of ethanol in 0.10 cc injection volume, that is a 0.001% solution, gave a discernible peak (of 0.10 mv) on the chromatogram. Using 3.00 cc of blood in the distillation this corresponded to a 10 mg % blood alcohol concentration. Column #1 gave the better ethanol peaks with respect to symmetry and sensitivity but failed to separate ethanol from formaldehyde and ethanol from methanol. Column #2 separated the binary mixtures as shown in Fig. 2. The separation of ethanol and methanol eliminates the necessity of separate methanol tests (6).

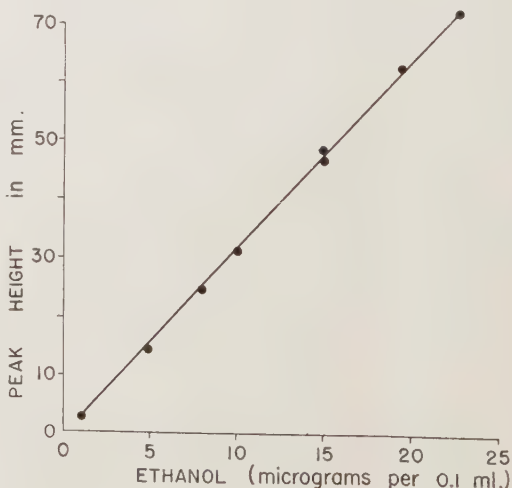


FIG. 1. Direct calibration of ethanol in 0.1 ml of water using column #1. Carrier gas, helium; flow rate, 20 cc per minute; column pressure, 1 psi, and temp., 106°C.

* Technical assistance by Carolyn S. Spaeth and Sandra Moulton is sincerely appreciated.

TABLE I. Recovery of Added Ethanol from Blood by Vapor Phase Chromatography Method Using Column 1.

| Ethanol added | Ethanol recovered* | Error | Corrected ethanol error† |
|------------------|--------------------|-------|--------------------------|
| (mg/cc of blood) | | | |
| .10 | .10 | .00 | .00 |
| .40 | .45 | -.05 | +.05 |
| .40 | .40 | .00 | .00 |
| .50 | .50 | .00 | +.05 |
| .50 | .45 | -.05 | .00 |
| .65 | .60 | -.05 | .00 |
| 1.00 | .95 | -.05 | .00 |
| 1.50 | 1.40 | -.10 | -.05 |
| 1.50 | 1.45 | -.05 | .00 |
| 1.50 | 1.55 | +.05 | +.10 |
| 2.00 | 1.95 | -.05 | +.05 |
| 3.00 | 2.90 | -.10 | +.05 |

* Significant to .05 mg.

† Measured from theoretical 95% recovery from alcohol-water azeotrope.

This method provides a more rapid means of performing blood alcohols on embalmed blood as compared to the existing procedures of distilling with aldehyde and ketone reagents(1,5). Although using 0.1 cc of H₂O overloaded the columns, that is the flow became irregular, no adverse effects on the glycol columns resulted. Gas chromatographic alcohol values and Widmark alcohol values on the same non-alcoholic putrified blood samples were compared. The reducing substances

(30-60 mg %)(1) as determined by the Widmark oxidation test on the slightly to moderately putrified blood did not add to the gas chromatographic blood alcohol value more than 5 mg %.

The two glycol columns also separated paraldehyde from binary mixtures of 100 mg of water and 20 μ g of the following substances: ether, acetone, acetaldehyde. Two micrograms of hydrogen sulfide gas were detected in blood distillates in 1 M 20:80 TCP-firebrick column at 94° and a 10 cc per minute helium flow. By the use of other columns additional volatile compounds may be determined. Preliminary investigation has shown that one microgram of carbon monoxide in air samples gave a millivolt recorder response in a 1 M Linde Molecular Sieve 13 X column at room temperature and 40 cc He/min flow. 10 μ g carbon tetrachloride and chloroform (from alkaline distillation of chloral hydrate) in 0.1 cc distillate were detected on a 20:80 triethylene glycol, firebrick column at temperature 78°C and flow of 30 cc He/min.

Summary. By fractional distillation of 1 to 3 cc of blood and gas chromatographic analysis of the distillate in glycol columns, blood ethanol concentrations were determined to an accuracy of 5 mg % and to a sensitivity of 10 mg % in the presence of methanol, formaldehyde and putrified blood. Microgram amounts of hydrogen sulfide, ether, acetone, acetaldehyde, chloroform and carbon tetrachloride were also identified in aqueous solutions.

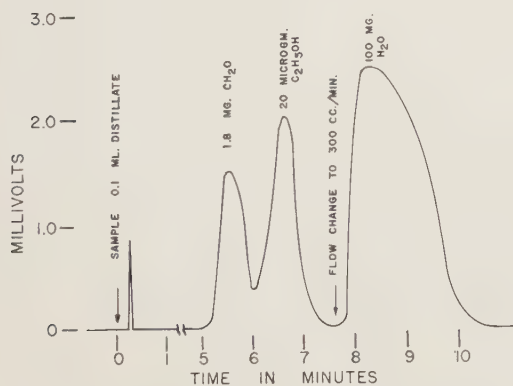


FIG. 2. Chromatogram of ethanol-formaldehyde-water using column #2; carrier gas, helium; initial flow rate, 20 cc per min; temperature, 84°C; sensitivity, 1 for aldehyde and alcohol; sensitivity, 1/128 for water.

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Effect of Chlorpromazine on Number of Mast Cells and of Certain Formed Elements of Blood. (23702)

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Recently we have suggested(1) that some of the hypothermic effects of chlorpromazine could be associated with the liberation of a variety of substances, such as histamine, serotonin and adrenaline, which individually will cause some drop in body temperature(1,2,3, 4). Some of these substances have been found in significant amounts in mast cells(5) and in some blood cells(6). The effect of chlorpromazine on mast cells and blood cells was the primary concern of the present study. Since the response to chlorpromazine is greatly reduced in acclimatized animals(1), and because of the reported changes in the number of mast cells of the same animals(7), the effect of chlorpromazine on mast cells of animals exposed to cold for varied periods of time was also investigated.

Methods. Male albino rats weighing about 300 g were used in all experiments. Chlorpromazine (10 mg/kg) was always injected intraperitoneally.* In a first experiment 1 group of 6 animals was used. Blood counts were made at 0, 4, 24 and 48 hr after injection of chlorpromazine. Blood samples were obtained by cutting the tip of the tail. The eosinophiles and total leucocytes were counted by using Henneman's method(8) and the erythrocytes and platelets, by the Rees-Ecker method(9). In a second experiment 4 groups of 6 rats were used. The first group served as control, the second received one injection of chlorpromazine, the third group, 5 injections of the same substance, and the fourth group, 5 saline injections. In the last 2 groups the injections were given at a rate of one per day. Finally in a third experiment 4 groups of rats were exposed to cold (6°C) for 0, 1, 7 and 28 days respectively. At the end of the cold exposure the animals were main-

tained at 24°C for 1 day before receiving chlorpromazine. In the last 2 experiments the mast cells in the intervacular area of the mesentery were examined 24 hr after the last injection in a manner described previously(7).

Results. Table I shows that 5 saline injections have little effect on the number of mast cells of the mesentery whereas a single injection of chlorpromazine reduces the number of these cells. This effect is greatly increased with 5 injections of chlorpromazine. The same table shows that in the preliminary stages of acclimatization (1 and 7 days) the response of mast cells to chlorpromazine is the same as the one observed in control animals. However, after 4 weeks in the cold chlorpromazine has very little effect on the number of mast cells. Table II shows that a single injection of chlorpromazine causes a very large drop in the eosinophile, leucocyte and platelet counts at 4 and 24 hr after the injection. After 48 hr a return towards normal values is observed in these counts although the initial levels have not been reached yet in the eosinophiles and platelets. A small transitory drop is observed in the number of erythrocytes. Measurements of rectal temperature have shown a marked drop in the first 2 hours after chlorpromazine injection, followed by a rapid return towards normal levels after 4 and 6 hours. Twenty-four and 48 hr after chlorpromazine injection the rectal temperature is back to normal.

Discussion. The large drop in the number of mast cells, eosinophiles, leucocytes and platelets caused by chlorpromazine is regarded as additional evidence to our previous suggestion that the hypothermic effect of chlorpromazine can be associated with the release of a variety of substances from either mast cells or blood elements(1). In the light of this hypothesis, the results obtained in acclimatized animals become somewhat easier

* Chlorpromazine was graciously supplied by Smith, Kline and French under their trade name of Thorazine.

TABLE I. Effect of Chlorpromazine on Mast Cells of the Intervascular Region of the Mesentery of Animals Exposed to Cold (6°C) for Various Periods of Time.

| Treatment | 0 | 5 saline | 1 CPZ* | 5 CPZ | 1 CPZ | 1 CPZ | 1 CPZ |
|--|------------|----------|----------|---------|----------|----------|----------|
| Length of exposure at 6°C before inj. of CPZ, days | 0 | 0 | 0 | 0 | 1 | 7 | 28 |
| Mast cells in mesentery (per 3 mm ²) | 304 ± 104† | 254 ± 34 | 160 ± 26 | 60 ± 14 | 172 ± 30 | 160 ± 60 | 252 ± 50 |
| P value‡ | | >0.3 | .02 | <.001 | .02 | .02 | 0.3 |

* CPZ = Chlorpromazine (10 mg/kg).
 † Stand. dev.

‡ P is calculated by comparing untreated group with the others.

TABLE II. Effect of Chlorpromazine (10 mg/kg) on Certain Formed Elements in the Blood of Normal Rats.

| Time after inj. (hr) | Eosinophiles | Leucocytes | Platelets | Erythrocytes | Rectal temp. (°C) |
|----------------------|--------------|------------|-----------|--------------|-------------------|
| | | | (× 1000) | (× 1000) | |
| 0 | 160 | 17,180 | 652 | 8,630 | 36.8 |
| 2 | | | | | 32.5 |
| 4 | 40 | 10,180 | 338 | 8,270 | 34.2 |
| 6 | | | | | 36.0 |
| 24 | 30 | 12,630 | 348 | 8,700 | 36.6 |
| 48 | 100 | 16,740 | 501 | 8,170 | 36.5 |

to interpret. Indeed, the very small destruction of mast cells caused by chlorpromazine in acclimatized animals could explain the reduced hypothermic effect of this drug under these conditions. This may not constitute the complete answer since the hypothermic effect of serotonin and histamine, which are products of secretion of mast cells, is also reduced in acclimatized animals(7).

Recently a large drop in platelet and leucocyte counts was reported in hypothermic dogs(10). Since these blood elements returned to normal levels as soon as the body temperature of the animals returned to normal, it was concluded that lowering of body temperature causes a sequestration rather than destruction of these blood cells. In our study on rats, however, the body temperature, after a drop of 4.3°C within the first 2 hours following the injection of chlorpromazine, has almost returned to normal levels in 6 hours. Consequently the fact that the blood cells studied are still at very low levels even when the body temperature has long returned to normal seems to indicate that the effect of chlorpromazine on certain blood cells is not a result of the hypothermic effect of this drug.

Summary. A single injection of chlorpromazine was shown to cause a large drop in eosinophiles, leucocytes and platelets in the

blood and in the mast cells of the mesentery. The effect of this drug on mast cells is negligible in cold acclimatized animals. This finding is associated with the smaller hypothermic effect of chlorpromazine in acclimatized animals than in control animals. It is suggested that the effect of chlorpromazine on formed elements of the blood and mast cells in the rat is not a direct result of hypothermia.

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Amino Acid Composition of Extracellular Protein from Six *Mycobacteria*.^{*} (23703)

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The amino acid composition of defatted mycobacterial cells has been reported in an earlier communication from this laboratory (1). In continuing these investigations we have now determined the amino acid composition of the extracellular protein prepared by a standardized procedure from 6 strains of mycobacteria.

Methods. *Mycobacterium tuberculosis* var. *hominis* 599, *M. tuberculosis* var. *avium*, *M. tuberculosis* var. *bovis*, *Mycobacterium phlei* (336/289B), *Mycobacterium ranae*, and *Mycobacterium smegmatis*, the same strains as those employed previously (1), were maintained throughout this study on the egg medium of Steenken and Smith (2). Cultures for protein production were grown on a modification of Long's medium (3) prepared as follows. DL-Malic acid 100 g, 10% ammonium hydroxide 400 ml, potassium dihydrogen phosphate 30 g, sodium chloride 20 g, magnesium sulfate 10 g, and ferric ammonium citrate 0.4 g, were dissolved in 9 l of distilled water. The solution was filtered, and the filtrate adjusted to pH 7.2 with 3N hydrochloric acid, was sterilized in 1 l Roux bottles, 180 ml per bottle, by autoclaving for 20 minutes at 121°. Prior to inoculation each bottle was supplemented with 20 ml of sterile 50% glucose solution. Inocula were prepared by seeding one or two such bottles with growth from the stock cultures. The remaining bottles, in each case, were inoculated from the fresh pellicles of the inoculum cultures. *M. smegmatis* was grown in a 15 l batch, *M. tuberculosis* var. *avium* in a 25 l batch and the other organism in 10 l

batches. The inoculated media were incubated at 37° for the following lengths of time: *M. ranae* 14 days, *M. phlei* 38-43 days, *M. tuberculosis* var. *hominis* 30 days, *M. tuberculosis* var. *bovis* 115 days, *M. tuberculosis* var. *avium* 40 days (10 liters) and 142 days (15 liters), and *M. smegmatis* 31-43 days. The cultures were clarified by Seitz filtration, and the filtrate concentrated 5 to 10 fold by evaporation under reduced pressure, were acidified by adding 50% trichloroacetic acid to a final concentration of 5%. After holding the latter solutions at least 24 hours at 4° the precipitated proteins were removed by centrifugation and redissolved in distilled water (a few drops of 3N sodium hydroxide were added to effect complete solution). The protein solutions were dialyzed against distilled water, treated with an equal volume of saturated ammonium sulfate solution, and allowed to stand 12 to 24 hours at 4°. The precipitate was collected by centrifugation and dissolved in distilled water. The procedure of precipitation in 50% ammonium sulfate solution was repeated, and the final precipitates, dissolved in distilled water, were dialyzed against distilled water for 4 days at 4°. The protein solutions were filtered and lyophilized. This procedure yielded the following amounts of lyophilized extracellular mycobacterial protein: *M. ranae* 685 mg, *M. tuberculosis* var. *hominis* 1.50 g, *M. tuberculosis* var. *bovis* 650 mg, *M. smegmatis* 700 mg, *M. phlei* 2.10 g, and *M. tuberculosis* var. *avium* 175 mg. The preparations were analyzed for moisture by drying them to constant weight in a vacuum oven at 56°, for ash by igniting them to constant weight in a closed crucible with a Fisher burner,† for nitrogen by the semi-micro Kjeldahl procedure, and for amino acids by microbiological assay methods. The samples for amino acid analyses were hydrolyzed by refluxing them

^{*} Paper 121. The material presented here has been taken in part from a thesis by Sarah L. Lovett, submitted in partial fulfillment of the requirements for degree Master of Science, Jan., 1956. This work was supported by grants from Amer. Trudeau Soc., the Los Angeles County Tuberculosis and Health Assn. and the University of California. The authors are indebted to Evelyn Brown, Audree Fowler and Ben Ginsberg for technical assistance.

† Moisture and ash analyses were carried out by Heather King.

TABLE I. Percentages of Moisture, Ash, Total Nitrogen* and Amino Acid Nitrogen† in Mycobacterial Proteins.

| Constituent | —In extracellular protein— | | | | | | | | | In dried, defatted cells |
|---------------------|----------------------------|---------------------------|-------------------------------|------------------------------|----------------|------|------------------|------|----------------|-----------------------------|
| | <i>M.</i> <i>phlei</i> | <i>M.</i> <i>ranae</i> | <i>M.</i> <i>smegmatis</i> | <i>M. tuberculosis</i> var.— | | | | | | |
| | | | | <i>avium</i> | <i>—bovis—</i> | | <i>—hominis—</i> | | | |
| | (%) | | | | | | | | | |
| | | | | | | ‡ | | § | | |
| Moisture | 6.7 | 5.7 | 6.4 | 3.7 | 5.6 | | 6.0 | | | 4.9 (1.9– 7.0) |
| Ash | 2.2 | 12.9 | 5.2 | 17.3 | 1.0 | | 1.6 | | | 3.9 (1.9– 7.8) |
| Nitrogen | 15.3 | 14.7 | 14.3 | 13.0 | 12.6 | | 15.3 | | | 10.2 (7.0–12.0) |
| Glutamic acid | 13.3 | 12.5 | 12.5 | 10.6 | 13.5 | 8.0 | 12.1 | 8.7 | | 6.2 (5.7– 9.6) |
| Alanine | 10.3 | 8.1 | 11.8 | 8.9 | 10.0 | 13.2 | 10.1 | 9.2 | | 9.6 (8.5–11.5) |
| Aspartic acid | 9.9 | 8.3 | 10.7 | 9.0 | 9.3 | 7.9 | 10.9 | 8.0 | 8.1 (6.7–13.1) | 4.4 (4.1– 4.8) |
| Leucine | 10.1 | 7.8 | 8.6 | 8.9 | 9.4 | 6.3 | 9.0 | 6.3 | | 5.8 (5.3– 6.3) |
| Valine | 8.1 | 6.6 | 8.2 | 6.5 | 8.4 | 2.7 | 7.9 | 5.4 | | 4.9 (4.4– 5.3) |
| Arginine | 7.5 | 6.0 | 5.9 | 5.7 | 7.3 | 15.9 | 6.4 | 15.6 | 4.8 (3.1– 5.8) | 15.4 (12.8–17.2) |
| Isoleucine | 5.3 | 3.9 | 4.7 | 4.5 | 5.1 | 4.6 | 4.8 | 4.3 | | 2.9 (2.7– 3.0) |
| Lysine | 5.0 | 3.6 | 5.2 | 4.6 | 4.6 | 6.0 | 4.5 | 4.1 | 4.1 (3.0–10.4) | 4.5 (3.5– 5.5) |
| Phenylalanine | 4.6 | 3.8 | 4.4 | 3.8 | 3.7 | 2.1 | 4.7 | 2.2 | 1.9 (.8– 3.8) | 1.6 (1.5– 1.7) |
| Serine | 3.0 | 3.6 | 4.1 | 3.7 | 4.1 | 4.3 | 5.0 | 3.1 | 3.8 (3.4– 5.7) | 2.8 (2.4– 3.1) |
| Tyrosine | 4.2 | 3.4 | 4.0 | 4.2 | 3.1 | 1.8 | 3.5 | 1.6 | | 1.1 (1.0– 1.2) |
| Histidine | 2.5 | 1.7 | 2.0 | 2.2 | 2.0 | 3.5 | 2.2 | 4.5 | 2.9 (2.0– 4.3) | 3.2 (2.8– 3.5) |
| Methionine | 2.0 | 1.7 | 1.6 | 1.3 | 1.6 | 1.2 | 1.8 | 1.3 | | .9 (.8– 1.0) |
| Amino acid total | 85.8 | 70.9 | 83.6 | 73.2 | 82.3 | 78.5 | 83.7 | 74.3 | | 64.0 (59.6–65.9) |

* Calculated for moisture and ash-free material.

† Calculated as % of total nitrogen. Literature data from other than the authors' laboratory are given in italics.

‡ Data of Jones *et al.*(7) for bovine tuberculin prepared by precipitation of culture filtrate at pH 4.95.§ Data of Jones *et al.*(7) for human tuberculin purified by a series of precipitations at pH 3.8 to 5.0 (material precipitating at pH 5.0 was excluded and final product was precipitated at pH 4.82).

|| Median and range of values of Seibert(8) for 5 tuberculin fractions obtained by alcohol precipitation, ammonium sulfate precipitation and urea extraction. Similar values reported by Nishihara(9) for a single human tuberculin preparation are omitted because they could not be presented on a comparable basis.

¶ Median and range of values of Ginsberg *et al.*(1) for dried defatted cells of 11 strains of mycobacteria (4 species), including 6 strains employed in the present study.

in 3*N* hydrochloric acid for 24 hours. Alanine was determined with *Leuconostoc citrovorum*(4), serine with *Lactobacillus casei*,† methionine with *Leuconostoc mesenteroides* P-60(5), and all other amino acids with the organisms and methods employed previously (6).

Results. Moisture, ash, total nitrogen, and amino acid nitrogen content of the extracellular mycobacterial proteins are given in Table I. Analogous results from other laboratories (7,8) and the earlier data from this laboratory on dried, defatted mycobacterial cells(1) are included in the table for convenience of comparing them with the present values. It may be seen (Table I) that the authors' 6 extracellular protein preparations obtained by a uniform procedure from different mycobac-

teria are quite similar in amino acid composition to each other, albeit, markedly different in this respect from the preparations of Jones *et al.*(7) obtained from closely related strains by a somewhat different procedure. The principal differences between the authors' preparations and those of Jones *et al.* are that the latter contained a much higher percentage of arginine nitrogen and a much lower percentage of glutamic acid nitrogen than the former. Smaller, but still significant differences in content of aspartic acid, leucine, valine, phenylalanine, tyrosine and histidine are also apparent. It appears, therefore, that different protein fractions derived from a single culture are generally less similar in composition than uniform fractions from different species, and this conclusion is borne out by the data of Seibert(8) (Table I) for 5 tuberculin fractions, all from *M. tuberculosis* var. *hominis*.

† Unpublished method of Camien and Dunn.

It may be seen that the spread of amino acid values for these five homologous tuberculins is in every case considerably greater than the corresponding spread of values for the authors' uniform preparations from six different strains, representing four mycobacterial species.

The extracellular proteins prepared by the authors' procedure are apparently quite different in amino acid composition from the dried, defatted cells of the strains from which they were derived. The arginine content, in particular, is much higher in the cells than in the extracellular material. Histidine, likewise, is appreciably higher in the cells. Glutamic acid, aspartic acid, leucine, valine, isoleucine, phenylalanine, tyrosine and methionine, on the other hand, are of significantly greater abundance in the extracellular proteins than in the corresponding preparations of cellular material.

Summary. Extracellular protein has been

obtained from 6 strains of mycobacteria (4 species) by a uniform procedure. The amino acid composition of the protein preparations is presented.

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Effect of Modification of Insulin on Its Degradation and Biological Activity.* (23704)

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Studies on the degradation of insulin labeled with I¹³¹ have shown that it is rapidly attacked by an enzyme or enzymes present in slices and homogenates of liver, kidney, pancreas, spleen, adipose tissue, and muscle(1-3). A large number of inorganic and organic compounds have been shown to inhibit insulin-I¹³¹ degradation in extracts of these tissues (4-7). The unusually high rate of insulin-I¹³¹ degradation observed after its injection into intact animals(8) poses many interesting questions for speculation, such as: Is the degradation linked in any way to the biological

activity of insulin or are the two processes, degradation and biological action, independent of one another? In an attempt to gain information on this aspect of the problem, we have carried out a series of experiments in which the insulin-I¹³¹ molecule was modified by reagents which react with one or more groups of the insulin molecule. The modified insulins were injected into eviscerated-nephrectomized rabbits and their rate of degradation was measured. The biological activity of the injected materials was determined simultaneously.

Procedure. General treatment of animals and determination of presence of biological activity in modified insulins. Adult female rabbits were eviscerated-nephrectomized and maintained as previously described(9). The plasma sugar was kept at a normal concen-

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tration by the constant injection of glucose. Rate of glucose injection was guided by frequent determination of the plasma sugar concentration. Glucose utilization was calculated from the quantity of glucose infused. Non-insulinized eviscerated - nephrectomized rabbits require from 140 to 210 mg/kg/hour of glucose to maintain a normal blood sugar level. When insulin is given in excess of 0.2 unit/kg the glucose utilization increases to 350 to 500 mg/kg/hour, a maximal rate for this preparation. In these experiments no attempt was made to evaluate the presence of insulin in amounts less than the equivalent of 0.2 unit/kg. *Insulin I¹³¹ degradation assay.* At standard time intervals after injection of the insulin-I¹³¹ derivatives, 2.5 ml blood samples were removed in oxalated syringes, centrifuged, and 0.5 ml aliquots of plasma precipitated with 10% trichloroacetic acid (TCA). The radioactivity in an aliquot of the plasma and in TCA supernatant was measured with an Atomic Instrument Co. Well-type scintillation counter of 35% efficiency, and the degradation was expressed as the per cent of total I¹³¹ soluble in 10% TCA. *Preparation of insulin derivatives.* Insulin-I¹³¹ was purchased from the Abbott Laboratories and contained one atom of I¹³¹ per mole of insulin. In the preparation of all insulin derivatives, 10 mg of crystalline zinc insulin (250 units) was mixed with approximately 0.025 mg of insulin-I¹³¹. This quantity of insulin-I¹³¹ was sufficient to give 30 to 50 million counts/minute in our well-type counter. Five % Na₂CO₃ was added until the crystalline zinc insulin was in solution, and the various insulin derivatives were prepared by treatment with the following reagents: *Diazobenzene Sulfonic Acid* was coupled to insulin by the procedure of Reiner and Lang(10). The diazotized product was pale yellow at pH 7.0, and was reprecipitated by lowering the pH to 5.5. A maximum of 3 radicals of diazotized sulfanilic acid was coupled to the insulin molecule. Aryldiazonium chlorides preferentially react with the aromatic ring of tyrosine and the heterocyclic ring of histidine(11). *Phenylisocyanate.* The urethan derivative of insulin was made according to the method of Fraenkel-Conrat and Fraenkel-Conrat(12). Phe-

nylisocyanate reacts with amino-, imidazol-, and phenolic groups of insulin under the conditions used. *Methanol.* Carboxyl groups were esterified with acidic methanol by the method of Charles and Scott(13). Esterification of macromolecular polyacids at room temperature by mineral acid catalysis has been well demonstrated. *2,4-Dinitrofluorobenzene* was reacted with insulin under the conditions of Sanger(14). The insoluble yellow product was dissolved by shaking repeatedly with dilute alkali, pH 10, at 50°. Under the conditions used, 2,4-dinitrofluorobenzene reacts with amino-, phenolic-hydroxyl-, thio-, and possibly with imidazole groups. *Formaldehyde.* Insulin was treated with formaldehyde for 56 hours at room temperature and acid pH as described by Fraenkel-Conrat and Fraenkel-Conrat(12). The solution was dialyzed against distilled water at 0° for twenty-four hours, and the pH adjusted to 7.0 with dilute NaOH. *Bis-diazobenzidine.* The insulin solution was adjusted to pH 10 and chilled in an ice bath; 2.5 mg of bis-diazobenzidine was added slowly with stirring, and the resulting deep-red solution was dialyzed overnight against distilled water at 0-4°. *Bis-diazobenzidine-Albumin.* 10 mg of human serum albumin in saline was added to the insulin solution. The pH was adjusted to 8.0 with dilute NaOH and the solution chilled in an ice bath. Twenty mg of bis-diazobenzidine was added at a rate of 0.4 mg every 5 minutes. The albumin-insulin complex was dialyzed for 24 hours against distilled water. The precipitate which formed during dialysis was re-dissolved by raising the pH to 8.0. The solution was dark brown at this point. The

formation of $\text{-NH-} \text{---} \text{C}_6\text{H}_4 \text{---} \text{C}_6\text{H}_4 \text{---} \text{NH-}$ link-

ages between proteins and cells constitutes the crosslinking bridge(15). *Sodium Hypochlorite.* Sodium hypochlorite containing 5% available chlorine was added to an alkaline solution of insulin, pH 10, in a ratio of 0.1 ml sodium hypochlorite per 10 mg insulin. Oxidation was complete in 3 minutes, and the material was dialyzed against distilled water at 0-4°. The solution was pale yellow at pH 7.0. This reaction has been

used to remove the guanidyl groups from collagen by Highberger and Salcedo(16). *Hydrogen peroxide*. Insulin was oxidized with 30% H_2O_2 by the procedure of Sanger and Thompson(17). The oxidized insulin was dialyzed for 24 hours at $0^\circ-4^\circ$, to remove excess hydrogen peroxide and formic acid.

Results. In Table I are data showing rate of glucose utilization and the rate of appearance of I^{131} in the TCA soluble fraction after injection of the insulin derivatives. Derivatives prepared with diazobenzene sulfonic acid, formaldehyde, or methanol were biologically active; all other derivatives showed impairment of insulin activity. No alteration in rate of degradation measured over a 4-hour period was found with derivatives prepared by treatment with diazobenzene sulfonic acid, formaldehyde, hydrogen peroxide or methanol; all other derivatives show a decrease in the rate of degradation. A summary of the relationship between biological activity, rate of degradation, and the reactive groups of insulin is given in Fig. 1.

Discussion. The use of the eviscerated-nephrectomized animal to study the degradation of insulin and its derivatives has the advantage that this preparation represents predominantly muscle metabolism *in vivo*, which is an important site of insulin action. The rate of insulin- I^{131} degradation is also

much slower in this preparation in comparison with intact animals(8), so that it is possible to correlate the rate of degradation with biological activity.

A fact to be considered in the study of protein derivatives is the lack of specificity of group reagents. Reagents once considered specific for a functional group can now be shown to react with more than one such group(18). Although the reagents used in this study were chosen to react mainly with one major group, the possibility of side reactions with other groups is present, and these studies, therefore, do not represent a specific relationship between a functional group, degradation, and activity.

The failure to observe biological activity with 6 derivatives of insulin (reagent Nos. 4-9 in Table I) is of greater significance than the 3 derivatives (Nos. 1, 2, 3) which showed no inhibition. This results from the fact that the presence of 0.2 unit of unreacted insulin from the initial 250 units of insulin employed for treatment with diazosulfanilic acid, formaldehyde, or methanol would produce the insulin-like glucose utilization values reported in Table I.

It has been shown that reagents which react mainly with tyrosine or histidine residues impair neither insulin degradation nor insulin activity. Similarly, mild non-oxidative iodination does not appear to alter either enzymatic degradation or biological activity(8). Replacement reactions on the tyrosine and histidine residues are possible without producing detectable changes in activity or degradation.

The importance of the configuration of the insulin molecule to its biological activity and the rate of degradation is shown with reagents which affect the size of the insulin molecule, *viz.*, bis-diazobenzidine and bis-diazobenzidine-albumin. Although these reagents react mainly with the histidine and tyrosine residues—ones which do not appear to be essential for either degradation or activity—polymerization and coupling with albumin destroyed insulin degradation and activity.

The guanidyl group of arginine appears to be linked with both insulin activity and insulin degradation. Removal of this group




| REAGENT NUMBER | GROUP ALTERED | ACTIVITY | DEGRADATION |
|----------------|---|----------|-------------|
| 1, 4, 5 |  | + | + |
| 1, 4, 5 |  | + | + |
| 2, 4, 5 | -NH ₂ | + | + |
| 3 | -COOH | + | + |
| 9, 5 | -S-S- | - | + |
| 8 |  | - | - |

FIG. 1. Summary showing groups modified in the insulin molecule and its effect on biological activity and degradation. Plus indicates presence of biological activity in the derivative and no measurable decrease in its rate of degradation. Reagent numbers refer to the designations in Table I.

TABLE I. Data Showing Rate of Glucose Utilization and % of Plasma-I¹³¹ Soluble in 10% Trichloroacetic Acid (TCA) after Injection of Insulin Derivatives in Eviscerated-Nephrectomized Rabbits.

| Reagent No. | Reagent used | No. of exp.* | Gluc. used, mg/k/hr | % plasma-I ¹³¹ TCA soluble | | | | |
|-------------|--|--------------|---------------------|---------------------------------------|------|------|------|------|
| | | | | .5 hr | 1 hr | 2 hr | 3 hr | 4 hr |
| | Control, 250 u insulin-I ¹³¹ only | 8 | 390 | 12 | 24 | 36 | 45 | 60 |
| 1 | Diazobenzene sulfonic acid | 2 | 380 | 5 | 15 | 28 | 44 | 56 |
| 2 | Formaldehyde | 3 | 385 | 4 | 15 | 31 | 46 | 55 |
| 3 | Methanol | 1 | 355 | 6 | 14 | 32 | 51 | 61 |
| 4 | Phenylisocyanate | 2 | 210 | 4 | 7 | 7 | 22 | 46 |
| 5 | 2,4-dinitrofluorobenzene | 1 | 193 | .3 | 1 | 4 | 4 | 8 |
| 6 | Bis-diazobenzidine | 2 | 210 | .9 | 2 | 11 | 10 | |
| 7 | Bis-diazobenzidine-albumin | 2 | 220 | 5 | 9 | 13 | 14 | |
| 8 | Sodium hypochlorite | 3 | 140 | 10 | 11 | 12 | 12 | 15 |
| 9 | Hydrogen peroxide | 2 | 211 | 5 | | 30 | 43 | 60 |

* Avg values are shown when more than one exp. was carried out. All animals weighed approximately 1 kilo after evisceration.

with sodium hypochlorite markedly interfered with both activities. That this is not a result of oxidation of disulfide bonds is seen from a comparison of the results obtained with insulin-I¹³¹ treated with hydrogen peroxide. In this case biological activity was destroyed but insulin degradation was not altered.

It is apparent that reagents which interfere with insulin degradation also interfere with biological activity, and the chemical groups on the insulin molecule that are essential for increasing glucose utilization are similar to those necessary for the action of the destructive enzymes. However, the results with hydrogen peroxide in which insulin activity was destroyed but insulin degradation not altered, indicate that the degradation of insulin and the biological activity of insulin are not necessarily dependent on each other. These results thus support the contention of Elgee and Williams(19) that the degradation of insulin-I¹³¹ may occur independently of insulin action.

Summary. The biological activity and rate of degradation of 9 derivatives of insulin were studied in the eviscerated-nephrectomized rabbit. Derivatives of insulin made with methanol, diazotized sulfanilic acid, formaldehyde, or hydrogen peroxide, did not affect insulin degradation; those made with 2, 4 - dinitrofluorobenzene, bis - diazobenzidine, bis-diazobenzidine-albumin, and sodium hypochlorite impaired insulin degradation. Those reagents which interfered with the rate at which insulin was degraded also destroyed

the biological activity of insulin. However, the reactive groups on the insulin molecule which were essential for biological activity were not necessarily centers of attack by the destructive enzymes. It is concluded that degradation of insulin-I¹³¹ observed in physiological systems is not necessarily a function of insulin action on target tissues.

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Distribution of Sulfur in Regenerating Wound Tissue. (23705)

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Methionine and cystine supplementation have been shown to be equally effective in accelerating healing rate of wounds in rats fed low and high protein diets(1,2). Methionine and cystine + cysteine (hereafter referred to as cystine) content of wound tissue was reported to exceed that of corresponding unwounded skin tissue 4 to 31 days after injury (3). Attempts to detect sulfur-containing compounds other than protein-bound amino acids have revealed no non-protein-bound wound sulfur(3). It was tentatively concluded that increased demand for methionine or cystine following injury is due to requirement of regenerating tissue for these amino acids, *per se*, as structural units of proteins (or, in the case of methionine, also as precursor of cystine). Since regenerating wound tissue contains considerable quantity of sulfate-containing mucopolysaccharides(4,5) the sulfur of which may be a product of *in vivo* metabolism of methionine and cystine(6), the presence of mucopolysaccharide sulfate in wound tissue was studied. Glutathione has been increased at site of rapid cell division(7) such as accompanies wound tissue regeneration. Non-protein fractions of wound tissue were studied with alterations in content of the peptide. Finally, levels of free cystine and methionine, and amount of S^{35} recently injected as S^{35} -L-methionine present in all forms discussed was studied. Special micro-techniques were employed to detect minute amounts of methionine, cystine, and sulfate in protein and non-protein fractions of wound tissue. Rate of S^{35} uptake following administration of S^{35} -L-methionine was also studied, radio-

active tracers serving as the most sensitive available method for detection of the various compounds.

Methods. Sixteen individually caged, female, albino rats (180 ± 15 g) of Sprague-Dawley strain, receiving distilled water *ad lib.*, were maintained on a protein-free diet (3) throughout. After 5-day acclimation period, each animal received intraperitoneally 5.05×10^6 cpm of S^{35} -L-methionine (Abbott) in 0.25 ml saline. Two days later, they were anesthetized with pentobarbital and a standard 4 cm circular skin wound placed on the back directly below scapula(3). Wound tissues were removed from 8 animals on 4 and 6 days after injury, the animals then sacrificed by decapitation. Tissues were carefully removed, weighed, and frozen at -20° . The frozen tissues were ground in mortar with sand and transferred to centrifuge tubes by washing with approximately 10 ml of saline. Ten ml of aqueous 10% trichloroacetic acid solution (TCA) was added to precipitate wound protein, the tubes then being centrifuged at 2000 rpm at 5° for 10 minutes. The precipitate was washed once with 5 ml of 5% TCA and supernatant fluids combined, filtered through Whatman No. 2 paper and diluted to 35 ml. The protein fraction was hydrolyzed 20 hours in 12 ml of 20% HCl - 50% HCOOH mixture(8), then diluted to 25 ml and filtered. S^{35} radioactivity present as mucopolysaccharide sulfate was determined by direct precipitation from acid hydrolyzate as $BaSO_4$, after addition of carrier sulfate. Sulfate- S^{35} in supernatant fluid was determined similarly. Total S^{35} radioactivity was determined on aliquots of supernatant fluid

* Part of thesis for degree of Master of Science.

TABLE I. Results of Chemical Analysis of Various Sulfur-Containing Compounds in Fractionated Wound Tissues. Results are expressed on the basis of mg sulfur/g of wet tissue.

| Days after wounding | Mucopoly-saccharide | Methionine | Cystine |
|-------------------------|---------------------|-----------------|-------------------|
| Wound hydrolysate | | | |
| 4 | .171 \pm .033† | .470 \pm .115 | .548 \pm .061 |
| 6 | .186 \pm .012 | .374 \pm .057 | .532 \pm .063 |
| Wound supernatant fluid | | | |
| 4 | 0 | .0054* | .0606 \pm .0096 |
| 6 | 0 | .0048* | .0592 \pm .0051 |

* Samples were pooled prior to analyses.

† Stand. dev. of mean.

and protein hydrolyzate by precipitation as BaSO₄ after oxidation with an HNO₃-HClO₄ mixture(9) and addition of carrier sulfate. Cystine radioactivity was determined after precipitation of the cuprous mercaptide(10). Methionine radioactivity was taken as the difference: total hydrolyzate radioactivity—(cystine radioactivity + mucopolysaccharide radioactivity). All radioactivity measurements were made at infinite thickness with a Tracerlab Windowless Flow Counter. Free sulfate in supernatant fluid and mucopolysaccharide sulfate in the acid hydrolyzate were determined turbidimetrically(11). Methionine in protein hydrolyzates was determined colorimetrically by Horn's modification (12) of the Sullivan-McCarthy method, while Folin-Winterstein colorimetric method involving reduction of phospho-18-tungstic acid was employed to determine cystine(13). These colorimetric methods were adapted to micro-scale for determinations on supernatant fluid fraction. For methionine assay 0.5 ml distilled H₂O, 0.05 ml 5 N NaOH, 0.01 ml sodium nitroprusside, 0.10 ml of 3% glycine and 0.10 ml of 85% phosphoric acid were employed. The microcystine method involved 1.3 ml distilled H₂O, 1.3 ml saturated sodium bicarbonate, 0.4 ml phospho-18-tungstic acid (1:1, aqueous), and 0.2 ml 10% sodium sulfite. Both methods gave linear absorption curves in 0.01-0.10 mg range of amino acid. A group of 36 animals, maintained under previously described conditions, were injected with 3.88×10^6 cpm doses of S³⁵-L-methionine, given 3 cm circular skin wounds, and 6 animals sacrificed by decapitation at scheduled times after wounding. Ra-

dioactivity and chemical measurements were performed on unfractionated wound tissues after acid hydrolysis, to ascertain presence of radioactivity as a function of time.

Results and Discussion. Results of chemical analyses (Table I) indicate that, on fourth and sixth days after wounding, covering interval of maximal metabolic activity, as indicated by previous work(3), protein-bound cystine contains the largest amount of sulfur in the wound, protein-bound methionine 20% less, and mucopolysaccharides approximately 50% as much sulfur as does methionine. No free sulfate was detectable in the non-protein fraction, either radioactively or chemically. The non-protein fraction contains approximately 1/10th as much cystine and 1/100th as much methionine as the protein fraction.

These data indicate that, while comparatively little sulfur is present in wound tissue in the supernatant fluid fraction, a considerable amount is present as mucopolysaccharides. As data in Table II indicate, the specific activity† of wound protein-bound cystine and methionine is much higher than that of mucopolysaccharide sulfur. This indicates that, while considerable sulfur is present in wounds as mucopolysaccharide sulfate, relatively very little of sulfur recently introduced into the body as S³⁵-L-methionine has been incorporated into wound tissue mucopolysaccharide.

TABLE II. Specific Activity of S³⁵ in Various Fractions of Wound Tissue. Each rat received 5.05×10^6 cpm of S³⁵-L-methionine intraper. 48 hr prior to wounding.

| Days after wounding | Mucopoly-saccharide SO ₄ ⁻ | Methionine-S ³⁵ | Cystine-S ³⁵ |
|-------------------------|--|----------------------------|-------------------------|
| Wound hydrolysate | | | |
| 4 | 1.30 \pm .14* | 10.20 \pm 1.64 | 12.82 \pm 1.06 |
| 6 | 1.29 \pm .21 | 32.40 \pm 3.86 | 10.82 \pm .93 |
| Wound supernatant fluid | | | |
| 4 | 0 | 75.74 \pm 13.3 | 5.11 \pm .59 |
| 6 | 0 | 95.08 \pm 15.5 | 4.12 \pm .25 |

* Stand. dev. of mean.

† Specific activity =

$$\frac{(\text{cpm/aliquot})(\text{wt of animal (g)})(10)}{(\text{mg amino acid S/aliquot})(\text{dose in cpm})}$$

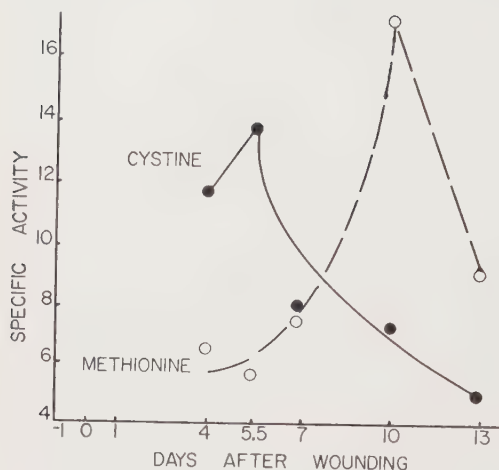


FIG. 1. Specific activity† of wound methionine and cystine as a function of time. Each point represents average of determinations on 6 rats. The more labile proteins of all animals were pre-labeled by injection of 3.88×10^6 cpm of S^{35} -L-methionine 2 days prior to wounding.

Variation of specific activity of wound methionine and cystine with time are shown in Fig. 1. Apparently rate of uptake of cystine- S^{35} by wound tissue, under conditions described, is more rapid than that of methionine, the specific activity of the former reaching a maximum at 5½ days post-injury while the latter reaches a peak at 10 days, thereafter dropping rapidly. The data in Table II from a separate experiment involving larger wounds, are consistent with these findings, protein-bound methionine specific activity rising between 4th and 6th days, while data for protein-bound cystine are considerably lower and approximately equal on 4th and 6th days.

Specific activity of the immediate precursor of the protein-bound amino acids, during period when specific activity is rising, must be higher than that of the material into which it is incorporated. While evidence indicates that this is true for the immediate precursor of protein methionine—free wound methionine—the specific activity of non-protein cystine is lower than that of the protein-bound form, on 4th and 6th days post-injury. That the specific activity of wound supernatant mercaptide-precipitable compounds on both days is lower than that of protein-bound cystine may suggest many explanations, among them,

either (a) all material in wound supernatant precipitable as cuprous mercaptide is not the immediate precursor of wound protein-bound cystine (this would be true if a large amount of material present were glutathione, the cysteine of which was supplied from sources different from those of highly radioactive wound cystine) or (b) wound tissue contains enzymes capable of converting methionine to cystine.

Our data substantiate the previous conclusion(3) that recently injected S^{35} -L-methionine is incorporated into wound tissue almost exclusively as protein-bound cystine and methionine, at least during first week of healing. Presence of elevated amounts of mucopolysaccharides and glutathione in wound tissue is indicated, but their low S^{35} contents indicate that little S^{35} from recently injected S^{35} -L-methionine has gone into formation of these compounds.

The seemingly anomalous results obtained here (Fig. 1) compared with previous publications(3) can be reconciled as follows. Under conditions of these experiments tissues were pre-labeled prior to injury and thus methionine-containing proteins would have to undergo catabolism before this amino acid was incorporated into wound protein. Therefore data in Fig. 1 would be expected to show a slow methionine incorporation over a long time; on the other hand, where one administered methionine to previously wounded animal, catabolism of tissue protein is not immediately involved in incorporation of labeled amino acid into wound tissue. Thus one would expect, and it was indeed found(3) that methionine was immediately taken up by wound tissue and rapidly turned over.

Summary. 1. Following pre-labeling of labile body proteins by injection of S^{35} -L-methionine and subsequent wounding, regenerating tissue was removed at 4 and 6 day intervals, fractionated, and assayed chemically and radioactively for bound and non-protein bound cystine and methionine, and for free and mucopolysaccharide sulfate. 2. Specific activity of unfractionated wound cystine and methionine was studied at intervals over 3 weeks. 3. The data indicate that, for the first 6 days post-injury, S^{35} administered as

S³⁵-L-methionine 2 days prior to wounding is incorporated into wound tissue in large measure as protein-bound methionine and cystine. 4. During the interval studied, administered methionine-S³⁵ is not a precursor for wound tissue mucopolysaccharide sulfate.

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Sensitivity of the Common Bile Duct to Acid Peptic Digestion.* (23706)

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Narrowing of the terminal biliary papilla as it enters the duodenal lumen has been observed in more than 50% of patients operated upon for gallstones(1). The causes for such narrowing are not apparent. The purpose of this study is to evaluate the sensitivity of the mucosa of the common bile duct to injury by acid-peptic juices, as the biliary component of the papilla does project into the duodenal lumen and could there be subjected to periodic submergence in gastric juice. If the bile duct epithelium proved sensitive to acid-peptic digestion, it is possible that erosive injury may play a role in bringing about the narrowing of the papilla observed so frequently in patients with gallstones. The recurrent stricture following choledochoduodenostomy could also find an explanation in such an occurrence. These considerations suggested the need to test the sensitivity of the mucosa of the common bile duct to acid-peptic digestion by perfusion with human gastric juice, with acid-pepsin mixtures, and with pancreatic juice and bile mixtures.

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Method. Adult cats were anesthetized with approximately 26 mg/kg body weight of sodium pentobarbital given intraperitoneally. Simultaneous perfusions of the esophagus and common bile duct were carried out. The sensitivity of the esophageal mucosa to injury by acid-peptic juice is well known(2) and concomitant perfusion of that organ could serve as a useful control. The gastric juice employed in the perfusion had been obtained from fasting patients by an inlying gastric tube attached to suction overnight for an 8-hour period from 11:00 p.m. to 7:00 a.m. Patients with peptic ulcer, cholelithiasis, as well as those with normal gastrointestinal tracts were studied. Gastric secretions were collected on ice and either analyzed immediately or frozen and stored below 0°C until used. The volume was measured and determinations of pH, free acid and pepsin were made. The pH was measured with the Northrup & Leeds glass electrode, free HCl was determined by titration with 0.1 N NaOH, and pepsin was determined by the hemoglobin substrate method of Anson(3). The gastric juice was adjusted to pH 1.6-1.7 at which acidity, optimal peptic activity is achieved. Commercial pepsin and acid solu-

tions were also made up and used as perfusing solutions in the same manner. In addition, human pancreatic juice as well as bile were perfused without changing the pH of the solution. Admixtures of human pancreatic juice and bile in a one to one ratio were then incubated for varying periods of time ($\frac{1}{2}$ to 48 hours) at 37°C prior to its use as an additional perfusing solution. The pancreatic juice was obtained from a patient with chronic pancreatitis whose pancreatic duct was cannulated for drainage and decompressive purposes at surgery. The specimens were collected on ice, cultures were obtained and the juice frozen immediately. Amylase, tryptic and lipase activities were determined before perfusion. All bile specimens used in the study were collected via a T-tube inserted at the time of common bile duct explorations. Perfusion of esophagus was performed as previously described(4). The common bile duct was perfused by cannulating the cystic duct near its entry into the common duct and ligating the major hepatic ducts proximally to preclude the retrograde flow of bile into the proximal reaches of the hepatic ducts. On occasions, one or both of the hepatic ducts were left open. The duodenum was then cannulated distal to the ampulla of Vater. In every instance the pylorus was occluded to prevent flow of gastric contents into the duodenum during the perfusion. The tubing carrying the perfusate to the cat was passed through a 37°C water bath to maintain constant temperature. Perfusion was carried out at a rate of 2-3 cc/min. (40-50 gtts/min.) under a pressure of 20 to 40 cm of water for 2 hours. Simultaneous perfusions of the esophagus and the common bile duct of the living anesthetized cat were carried out. The 2-hour end point in the esophagus has proved very practical in assessing the sensitivity of the esophageal mucosa to injury by digestive juices. Perfusions in the first 4 instances, however, were carried out for up to 20 hours. We had not anticipated finding the bile duct mucosa as sensitive to digestion by acid-peptic juice as the esophagus. The animals were then sacrificed and the degree of digestion produced graded zero to five +. (1 + = erosion of the mucosal epithelium; 2 + =

TABLE I.

| A. Source of gastric juice Patient's diagnosis | No. of cats | Actual perforation or 4+ digestion | |
|---|-------------|------------------------------------|-----------|
| | | CBD | Esophagus |
| Cholelithiasis | 6 | 3 | 2 |
| CBD stone | 1 | — | — |
| Duodenal ulcer | 18 | | |
| Pre-op. | 9 | 8 | 6 |
| Post-op. | 9 | 1 | 2 |
| Ca. of stomach | 1 | 0 | 1 |
| Miscellaneous | 4 | 1 | 0 |
| Total | 31 | 13 | 11 |
| B. Commercial pepsin sol. | 3 | 3 | 3 |
| HCl solution (0.1 N) | 3 | 0 | 0 |
| Human bile | 2 | 0 | 0 |
| Pancreatic juice | 2 | 0 | 0 |
| C. Human bile and pancreatic juice | | | |
| a Incubated $\frac{1}{2}$ hr | 3 | 0 | 0 |
| b | 12 | 0 | 0 |
| c | 24 | 0 | 0 |
| d | 36 | 0 | 0 |
| e | 48 | 0 | 0 |

erosion into the submucosa; 3 + = digestion into the muscular layers; 4 + = impending perforation with only serosa being intact; 5 + = perforation.)

Results. Over one-third of the animals studied had either severe digestion or perforation of the common bile duct during the perfusion period (Table I A). A like number of esophageal perfusions resulted in comparable degrees of digestion. Of 31 cats in which perfusions were carried out, actual perforation (5 +) or severe digestion (4 +) of the common duct was seen in 13 instances. Simultaneous esophageal perfusions performed demonstrated similar changes in 11 cats. In 3 animals perforations of both the common bile duct and esophagus occurred.

When the common duct was perfused with 0.1 N HCl, little damage was seen, but the addition of 1.2 mg/cc pepsin to the perfusate regularly produced severe digestion (Table I B).

Investigators have recently demonstrated that bile and pancreatic juice in a 1 to 1 mixture when incubated for 12-48 hours and perfused in the pancreatic duct produced a lethal hemorrhagic pancreatitis in 100% of the animals (dogs) studied(5). Such mixtures were also evaluated in this study since obstruction

of a common channel at the sphincter of Oddi is occasionally seen in patients with biliary tract disease. Perfusion of the common bile duct with this solution demonstrated relatively little digestion ranging from 0 to 2+ (Table I C) over the 2-hour period of the study. On one occasion a 3+ erosion was noted. Whether one or both of the major hepatic ducts were ligated did not appear to influence the degree of digestive changes.

Summary. 1. The glandular mucosa of common bile duct shows a sensitivity to acid-peptic digestion at least as great as that of the esophagus. 2. Admixture of bile and pancreatic juice incubated from 1/2 to 48 hours failed to demonstrate evidence of digestive injury to either the common bile duct or

the esophagus during a 2-hour period of perfusion. 3. There would appear to be a possibility that acid-peptic erosion of the biliary papilla may play a role in the genesis of stricture of the bile duct in some disease states.

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Tolerance and Withdrawal Hyperexcitability Induced in Mice by Chronic Administration of Phenaglycodol.* (23707)

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Several clinical reports(1-5) reveal that abrupt withdrawal of meprobamate (Miltown, Equanil) medication in patients who have ingested large doses of this drug may precipitate nervousness, insomnia, and convulsions. Work in our laboratories(6) indicates that tolerance and withdrawal hyperexcitability result from chronic administration of large doses of meprobamate to mice, as measured by the technic of McQuarrie and Fingl(7). Tolerance and withdrawal convulsions were also observed to occur after administration of large doses of this drug in dogs, by Essig and Ainslie(4). Since both meprobamate and phenaglycodol (Ultran) are substituted propanediols and have similar pharmacological activities in experimental animals(8,9), it was of interest to determine the effect of chronic administration of large doses of phenaglycodol and of its subsequent abrupt with-

drawal on central nervous excitability in mice. In the absence of positive evidence that this drug induces tolerance and physical dependence in man, it was anticipated that such a study might predict the effects of abrupt withdrawal of phenaglycodol in patients who have been chronically ingesting large doses. The results obtained provide the basis for this report.

Methods. Male albino mice (Carworth Farms, CF #1 strain, 21 to 32 g in weight) were randomly divided into 2 groups of 50 animals each. Phenaglycodol† was administered orally to one group as a 2 or 3% suspension in 6% acacia solution, in a total daily dose of 600 mg/kg (200 mg/kg at 7 a. m., 3 p. m., and 12 midnight) for 6 days. Because of the development of tolerance, following the 7 a. m. treatment on day 7 each dose was increased by 50% to make a total daily dose of 900 mg/kg; this higher daily

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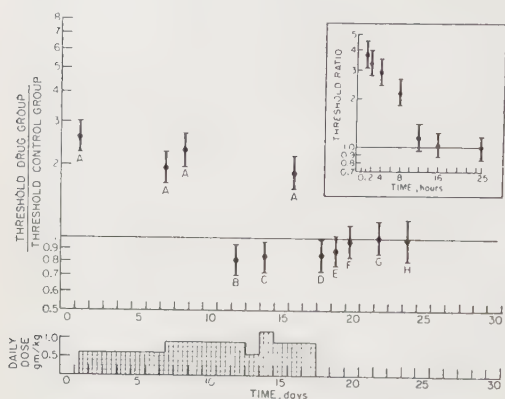


FIG. 1. Effects of chronic administration of phenaglycodol (Ultran) on threshold for low-frequency electroshock seizures in mice. Time in days is indicated on the abscissa. Seizure threshold ratio (threshold drug group/threshold control group) is shown on upper ordinate and total daily dose of phenaglycodol administered is shown below. Vertical bracketed lines indicate 95% fiducial limits. A, ratio 1 hour after a 7 a.m. dose; B, ratio 8 hours after the 11 p.m. dose; C, ratio 16 hours after the 3 p.m. dose. Following the 16-hour interval, the animals were given a double dose in order to maintain the average daily dose of 900 mg/kg. D, E, F, G, and H, ratios at 8, 32, 56, 104, and 152 hours, respectively, after the final dose of drug. The inset illustrates the duration (hours) of the effect of a single dose of phenaglycodol (300 mg/kg) on seizure threshold in nontolerant mice.

dosage was continued for 11 days, except as indicated in Fig. 1. The other group served as a control and was given the requisite volume of 6% acacia solution at the appropriate intervals. Low-frequency electroshock seizure threshold was determined for both groups at the times indicated in the legend to Fig. 1. Seizures were induced through corneal electrodes by means of a Grass stimulator (model S4B). The stimulus parameters employed were the same as those previously described (10), namely, unidirectional pulses of 0.2 msec. duration delivered for 3 seconds at a frequency of 6 pulses per second. The mice were shocked at various voltages which were selected by the staircase procedure (11), and the voltage required to evoke convulsions in 50% of each group was determined; the 95% fiducial limits were calculated by the method of Litchfield and Wilcoxon (12). Results are presented as the threshold ratio (threshold of drug group/threshold of control group). In a separate series of experiments, 80 mice were divided into 2 equal groups. One group was given orally 300 mg/kg of phenaglycodol, the

other group the requisite volume of 6% acacia solution; the seizure threshold of each group was determined 1 hour after drug administration. This procedure was repeated at intervals of 4 to 9 days in the same group of mice until the seizure threshold was established at 1, 2, 4, 8, 12, 16, and 25 hours after drug administration.

Results. Administration of a single 300 mg/kg dose of phenaglycodol to nontolerant animals increased the threshold 3.7-fold (see inset, Fig. 1), whereas, after treatment for 6 days with 600 mg/kg/day, and then for 11 days with 900 mg/kg/day, this drug increased the threshold only 1.9-fold (see A, day 16, Fig. 1). Thus, tolerance develops to the threshold-raising effect of phenaglycodol.

The figure also shows that, 8 hours after the 11 p.m. dose on day 11, 16 hours after the 3 p.m. dose on day 13, and 8 hours after the final dose on day 17 (see B, C, and D, Fig. 1), the seizure threshold in phenaglycodol-treated mice was only 0.81, 0.84, and 0.85, respectively, that of control animals. Since the seizure threshold 8 hours after administration of a single dose of 300 mg/kg to nontolerant animals was still 2.19-fold that of control mice (see inset, Fig. 1), the reduction in threshold observed in tolerant animals must be attributed to the chronic administration of phenaglycodol.

Discussion. Since the objectives of this study were to determine whether tolerance develops during chronic administration of phenaglycodol and whether abrupt withdrawal is followed by increased excitability of the central nervous system, only high dose levels of phenaglycodol were employed. The use of large doses is also justified on the basis that excessive amounts are taken by patients who abuse drugs and such quantities are usually necessary to demonstrate physical dependence on ethanol and barbiturates in man (13,14).

The data presented indicate that tolerance and withdrawal hyperexcitability follow chronic administration of large doses of phenaglycodol to mice. Tolerance is shown by the fact that the threshold-raising effect of phenaglycodol is reduced by 50% in animals receiving the drug chronically. Withdrawal hyperexcitability is shown by the fact that

8 hours after the 11 p. m. dose on day 11 and 8 hours after the final dose on day 17 (see B and D, Fig. 1), the seizure threshold in phenaglycodol-treated mice was only 0.81 and 0.85, respectively, that of control animals; a similar decrease in threshold was also observed 16 hours after the 3 p. m. dose on day 13 (see C, Fig. 1). Since seizure threshold is still elevated 8 hours after the single administration of this same dose of phenaglycodol to nontolerant animals, it must be concluded that the reduction in threshold represents withdrawal hyperexcitability attributable to the chronic administration of this agent.

Because of their common chemical derivation and similar pharmacological properties, it is of interest to compare meprobamate and phenaglycodol for ability to induce tolerance and withdrawal hyperexcitability in mice. With regard to tolerance, previous work in our laboratories(6) has shown that the threshold-raising effect of 300 mg/kg of meprobamate is reduced by at least 75% in animals treated for 6 days with 1200 mg/kg/day and for 10 days with 1800 mg/kg/day. The data presented in this study indicate that the threshold-raising effect of 300 mg/kg of phenaglycodol is reduced by 50% in animals treated for 6 days with 600 mg/kg/day and for 11 days with 900 mg/kg/day. With regard to withdrawal effects, hyperexcitability in mice following the chronic administration of meprobamate is detectable 4 and 8 hours after drug withdrawal and subsides within 28 hours(6). In the present studies, hyperexcitability in mice following chronic administration of phenaglycodol is detectable 8 and 16 hours after drug withdrawal and subsides within 32 hours. Thus, there is considerable similarity both in the extent of the tolerance developed during chronic administration of these agents and in the time course of the hyperexcitability following their abrupt withdrawal.

In view of the above observations, certain clinical implications require mention. The similarity in the withdrawal hyperexcitability induced in mice by the chronic administration of either phenaglycodol or meprobamate and

the evidence that continued ingestion of large doses of meprobamate can cause physical dependence in both animals and man(6,4) suggest that phenaglycodol should be held suspect, even though there have as yet been no clinical reports describing physical dependence to this drug. It is possible that patients taking large doses of the drug for extended periods of time may exhibit withdrawal effects which the physician may erroneously interpret as due to a return of pre-treatment symptoms. The drug should not be used for trivial complaints and the dose should not be excessive. Precautions should be taken in patients to minimize both the possibility of development of physical dependence on phenaglycodol and the possibility of a withdrawal syndrome. Thus, large doses of phenaglycodol should be avoided if possible, and the drug should be withdrawn slowly, rather than abruptly, from patients who have been ingesting large doses chronically.

These data and those previously reported for ethanol and meprobamate(7,6) indicate that the technics employed herein may be useful as a presumptive test for screening candidate drugs for physical dependence liability. Although preliminary data are in agreement with this suggestion, the predictive value of this test can only be determined after more laboratory and clinical results have been accumulated on a larger number of centrally active drugs

Summary. Tolerance and withdrawal hyperexcitability were observed to follow the chronic administration of large doses of phenaglycodol (Ultrán) in mice. Tolerance was shown by the fact that the threshold-raising effect of this agent is reduced by 50%, as measured by the low-frequency electroshock technic. Withdrawal hyperexcitability was shown by the fact that, 8 hours after the final dose of phenaglycodol, the seizure threshold is reduced by approximately 20%. The clinical implications of these observations are discussed.

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TABLE I. Incorporation of Adenine-8-C-14 into Nucleic Acids of Neural Axis of the Mature Cat.

| Exp. | Dose & route† ($\times 10^6$ cpm) | | Time interval | S.A.* | | | | |
|------|---------------------------------------|----|------------------|----------------------|----------------|-------------------|--------------------|------------|
| | | | | DNA | | | | |
| | | | | Cervical spinal cord | | | | |
| | | | | White matter | Gray matter | Spinal ganglia | Sp. nerve roots | Hindbrain‡ |
| CA 9 | 30.7 | IC | 1 hr | 14,650 | 3,260 | | | 1,270 |
| | 30.7 | IS | | 775 | 760 | | | 54 |
| 10 | 15.4 | IC | 2 | 61,000 | 3,520 | 1,700 | 21,200 | 5,720 |
| | | | | 433 | 117 | 230 | 820 | 237 |
| 8 | 15.4 | " | 4 | 12,500 | 3,380 | 2,440 | | 418 |
| | 15.4 | IS | | 593 | 945 | 960 | | 23 |
| 11 | 15.4 | IC | 24 | 81,000 | 39,200 | 10,600 | 88,400§ | 38,000 |
| | | | | 620 | 506 | 322 | 513§ | 261 |
| 12 | 15.4 | " | 46 | 21,600 | 3,610 | 3,270 | 56,300§ | 3,550 |
| | | | | 640 | 245 | 455 | 480§ | 51 |
| 3 | 15.4 | " | 9 days | 77,800 | 64,500 | | | 9,950 |
| | | | | 2,330 | 2,250 | | | 285 |
| 7 | 20.5 | " | 51 | 30,800 | 23,000 | 3,050 | | 30,000 |
| | | | | 24,500 | 12,800 | 2,660 | | 4,730 |
| | | | | 53,500 | 15,700 | 3,840 | 19,900 | |
| | | | | 38,200 | 14,900 | 2,250 | 17,000 | |
| 5 | 69 | " | 9 | 236,000 | 174,000 | | | 66,800 |
| | in 5 daily inj. | | | 48,300 | 31,500 | | | 2,020 |

* Specific activity (counts/min./mg nucleic acid). Nucleic acid was calculated from the absorbance at 260 $m\mu$.

† IC = intracisternal inj. IS = intraspinal inj. S.A. of adenine-8-C-14 = 15.4×10^6 cpm per mg.

‡ Medulla, pons and cerebellum.

§ Accuracy questionable because of small sample size.

|| Lumbosacral spinal cord.

was calculated from the formula $\mu\text{g PNA} = \frac{A_{260} \times V}{.0318}$ where A_{260} = absorbance at 260

$m\mu$ (1 cm path), V = volume to which aliquot is diluted and .0318 is the absorbance at 260 $m\mu$ of a hydrolyzed acidified solution of yeast PNA ($N = 15.5\%$, $P = 8.9\%$) containing 1 $\mu\text{g/ml}$. The amount of DNA plated for counting was calculated from the formula

$\mu\text{g DNA} = \frac{A_{260}}{.019} \times V$ where .019 is the ab-

sorbance at 260 $m\mu$ of a solution of purified calf thymus DNA ($N = 15.3\%$, $P = 9.1\%$) in .005 N NaOH containing one $\mu\text{g/ml}$. All samples were counted in a gas flow counter (Nuclear Model D 47 equipped with "micro-mil" end window) in conjunction with an automatic sample changer to give a standard error in counting of 3% or less; appropriate corrections for background, self-absorption of sample and counter efficiency were made in

calculating the specific activity (S.A.) of PNA and DNA.

Results. The results are presented in Tables I and II. It is evident that labeled adenine and orotic acid were rapidly incorporated into PNA in the central nervous system of the adult cat. The specific activity (S.A.) varied considerably from experiment to experiment; this may be attributable to unavoidable differences in local concentration of labeled compounds which may be affected by factors such as speed of injection, cerebrospinal fluid movement, diffusion away into the blood stream and others. However, the retention of labeled precursors, particularly adenine, in the nucleic acids of the neural axis for as long as 51 days is striking. In general, the DNA fraction was labeled only slightly as compared with PNA and could be caused by labeled contaminants, particularly PNA.‡ In animals receiving labeled precursors in

TABLE II. Incorporation of Orotic-6-C-14 Acid into Nucleic Acids of Neural Axis of the Mature Cat.

| Exp. | Dose & route† (× 10 ⁶ cpm) | | Time interval | S.A.* | | | | Hindbrain‡ |
|------|--|----|------------------|----------------------|----------------|--------------------|---------|------------|
| | | | | PNA | | Sp. nerve roots | | |
| | | | | DNA | | | | |
| | | | | Cervical spinal cord | | | | |
| | | | | White matter | Gray matter | Spinal ganglia | | |
| CO 6 | 7.3 | IC | 4.5 hr | 11,350 | 14,700 | | 6,550 | |
| | | | | 780 | 495 | | 89 | |
| 4 | 14.5 | " | 4 | 6,550 | 5,080 | 805 | 16,200 | |
| | " | IS | | 98 | 133 | 184 | 151 | |
| 7 | " | IC | 23 | 90,500 | 57,000 | 4,800 | 107,000 | |
| | | | | 375 | 485 | 485 | 400 | |
| 2 | 7.3 | " | 43 | 5,460 | 447 | 758§ | 15,300§ | |
| | | | | 143 | 154 | 506§ | 116§ | |
| 3 | 21.8 | " | 44 | 58,200 | 72,000 | | 16,000 | |
| | | | | 327 | 306 | | 31 | |
| 5 | 14.5 | " | 51 days | 5,250 | 5,670 | | 6,400 | |
| | " | IS | | 2,510 | 1,260 | | 1,960 | |
| | | | | 1,980 | 1,420 | 298 | 1,950 | |
| | | | | 1,010 | 1,310 | 300 | 1,620 | |
| 8 | 58.1 | IC | 15 | 105,500 | 118,500 | 5,870 | 82,300 | |
| | in 4 daily inj. | | | 11,850 | 2,620 | 371 | 2,870 | |
| | | | | | | | 85,000 | |
| | | | | | | | 1,780 | |

* Specific activity (counts/min./mg nucleic acid). Nucleic acid was calculated from the absorbance at 260 m μ .

† IC = intracisternal inj. IS = intraspinal inj. S.A. of orotic acid-6-C-14 = 7.3×10^6 cpm per mg.

‡ Medulla, pons and cerebellum.

§ Accuracy questionable because of small sample size.

|| Lumbosacral spinal cord.

relatively large doses by multiple daily injections and in animals surviving for 51 days following a single injection, the labeling of DNA is probably significant; in these experiments the specific activity of the DNA fraction approached the specific activity of PNA in many of the tissue samples (experiments CA7, CA5, CO5 and CO8). However, while these data suggest incorporation of labeled precursors into DNA under the conditions of these experiments, isolation and measurement of specific activity of constituent mononucleotides is required to prove such incorporation. It is particularly important to substantiate these findings because cell division probably does not occur to any significant degree in the central nervous system of healthy adult mammals(12) and DNA is widely believed to be metabolically stable in the absence of cell division(13). If this should prove to be true incorporation into DNA, it would follow that the labeled precursor was derived either directly or indirectly

from that previously incorporated into PNA. Work is underway to resolve this question.

The PNA of white matter of spinal cord exhibited consistently high S.A.; with adenine it always exceeded the S.A. of PNA derived from gray matter of the same segments. In some experiments the precursors appear to have been metabolized and fixed so rapidly as it diffused through the outer white matter that relatively little penetrated into the centrally placed spinal gray during the elapsed time. The S.A. of PNA from spinal nerve roots was also high; in sharp contrast to this, the S.A. of PNA of spinal ganglia was relatively low. The PNA of hindbrain was generally less active than that of cervical spinal cord. This is believed to be a reflection of local differences in movement of cerebrospinal fluid and diffusion distance in neural tissue rather than an intrinsic difference in nucleic acid incorporation in these two parts of the neural axis. In those experiments in which DNA may have been significantly labeled, *i.e.*,

long term and multiple injection experiments, *vide supra*, the distributional pattern followed closely that of PNA. The DNA fraction of spinal white was generally most active, that of spinal gray and spinal nerve roots somewhat less active and that of spinal ganglia least active.

The extremely rapid incorporation of labeled precursors into PNA and the prolonged retention of these precursors in PNA in the neural axis appear incongruous at first sight. However, these findings can be reconciled if it is assumed that the catabolic products are used over and over in the dynamic turnover of PNA. Indeed, the presence of a blood-brain barrier which greatly restricts penetration of nucleic acid precursors would make such conservation useful if not essential. The high turnover of PNA in spinal white matter indicates the occurrence of active nucleic acid metabolism in glial cells. This conclusion is supported by results of autoradiographic studies carried out in this laboratory(13), which reveal labeling of PNA of interfascicular oligodendrocytes in the white matter of spinal cord and brain. The same conclusion appears warranted for the glial and Schwann cells of the spinal nerve roots. Incorporation into PNA of glial cells and nerve cells in gray matter has been observed in autoradiographs (14). It is interesting to note that nuclear PNA becomes labeled some hours before cytoplasmic PNA in neurons within the central nervous system. Spinal ganglion neurons and surrounding satellite cells manifest a much less active incorporation of adenine or orotic acid into their PNA in autoradiographs. This agrees with the biochemical results and appears to be a significant metabolic difference between the multipolar neurons within the neuraxis and the unipolar spinal ganglion neurons.

There is much evidence of an association between PNA and protein synthesis; cells rich in PNA also actively synthesize protein(16). However, the form of this association is not yet understood. The abundance of PNA in neurons has been related to protein synthesis by Hyden(1,3). Histochemical and autoradiographic studies by others(17-20) have

demonstrated active incorporation of this labeled amino acid into nerve cell protein. Weiss and Hiscoe's demonstration(21) of a centrifugal flow of axoplasm in peripheral nerves of adult mammals suggests a purpose for protein synthesis by the neuron soma, namely, replacement of axoplasm dissipated in the peripheral termination or perhaps consumed throughout the entire extent of the axone. The active turnover of PNA in white matter has not been previously reported. Biochemical studies with S-35 methionine(15) have shown rapid incorporation into protein of spinal white matter in cats; in high resolution autoradiographs, this labeled protein was found chiefly in oligodendrocytes and in the glial septa enveloping myelin sheaths. Therefore, the interfascicular oligodendrocyte, as well as the nerve cell, appears to be a cellular site of active PNA and protein turnover. Since protein represents an important component of the myelin sheath(22), it is tempting to relate the nucleoprotein metabolism of interfascicular oligodendrocytes to myelin biosynthesis and to maintenance of the myelin sheaths in the mature animal. It is possible that neurological diseases characterized by demyelination reflect a disturbance in nucleoprotein metabolism of interfascicular oligodendrocytes.

Summary. 1. Adenine-8-C-14 and orotic-6-C-14 acid when injected intrathecally were rapidly incorporated into pentose nucleic acid of the central nervous system of mature cats. Once incorporated, these labeled precursors were retained for a relatively long time (51 days). 2. The pentose nucleic acid of spinal white matter and spinal nerve roots exhibited high specific activity, generally exceeding that of spinal gray matter and hindbrain. Spinal ganglia showed the lowest specific activity of pentose nucleic acid. 3. The deoxypentose nucleic acid showed insignificant activity in most experiments. When animals received repeated injections of labeled compounds and when they survived for longer periods of time (51 days), the deoxypentose nucleic acid fraction exhibited high activity; however, biochemical proof of incorporation is not yet available.

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α and β Lipoproteins and Serum Cholesterol Levels Following Administration of Unsaturated Fatty Acids. (23709)

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(Introduced by L. Freedman)

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Quantitative and qualitative alterations of ingested fat have been shown to regulate the composition of transported lipids in the human subject(1,2). Bronte-Stewart(3) postulated that atherosclerosis is possibly intensified by a dietary deficiency of essential fatty acids. The effect of lipotropic substances on dispersion of fat has been reported(3) and confirmed(4,5). The experimental data presented below indicate that serum alpha and beta lipoprotein and cholesterol levels are significantly modified by administration of essential fatty acids and lipotropic substances. The importance of these findings in the prevention of atherogenesis is discussed.

Material. Twenty-five patients, 16 males and 9 females, age 36 to 78, with clinical evidence of myocardial infarction, under our continuous observation for 4 to 5 years, were the subjects of this study. Controls were 20 subjects, ranging in age from 10 to 44 and one child of 3 (19 males and 2 females) with no

demonstrable cardiovascular disease. The experimental as well as the control subjects were encouraged to remain on a normal diet with no restriction of fat, cholesterol-containing or any other food.

Methods. Determinations were done monthly on fasting serum of controls, experimental subjects and on pooled serum from healthy blood donors. Each blood sample was analyzed by a modified electrophoretic method for lipoprotein and protein fractions and by chemical analysis for cholesterol. The experimental subjects had been previously studied on an unrestricted diet while receiving a lipotropic mixture* and results reported(6). The same diet and lipotropic mixture were continued but to them was added 3 g of saf-

* Daily intake comprised 1 g of choline, 1 g of dl-methionine, 750 mg of inositol, 18 μ g of vit. B₁₂ and 750 mg of desiccated liver, supplied as Methischol Capsules, courtesy of U. S. Vitamin Corp., N. Y. City.

TABLE I. α/β Lipoprotein Ratio in 21 Presumably Healthy Subjects and in Pooled Serum from Apparently Healthy Donors (Multiple Runs).

| Age | Sex | α/β lipoprotein ratio |
|----------------------------|-----|----------------------------------|
| 22 | ♂ | .58 |
| 29 | ♂ | .72 |
| 19 | ♀ | .67 |
| ? | ♀ | .47 |
| 10 | ♂ | .89 |
| 21 | ♂ | .69 |
| 22 | ♂ | .82 |
| 27 | ♂ | .52 |
| 44 | ♂ | .41 |
| 11 | ♂ | 1.10 |
| 23 | ♂ | .75 |
| 26 | ♂ | .41 |
| 33 | ♂ | .70 |
| 25 | ♂ | .78 |
| 11 | ♂ | .95 |
| 31 | ♂ | .56 |
| 3 | ♂ | .89 |
| ? | ♂ | .81 |
| 26 | ♂ | .82 |
| 28 | ♂ | .90 |
| 27 | ♂ | .95 |
| Avg | | .734 |
| Blood donors' pooled serum | | .62-.67 |

flower seed oil and 4 mg pyridoxine per day.[†] Maximum effect on fat metabolism had been achieved by the earlier regimen, and significant changes were considered to be due to the added safflower seed oil.

Results. Table I lists the values for controls. The range of alpha to beta ratio is from 0.41 to 1.10; the mean is 0.734. This mean differs only insignificantly from the mean of pooled serum. The technic error for the alpha to beta ratio appears to be 0.05. This has been repeatedly confirmed in several thousand electrophoretic separations.

Table II lists the alpha to beta lipoprotein ratio and total serum cholesterol before medication and after 12, 18, 24 and 30 week intervals. The average alpha to beta lipoprotein ratio before administration of the safflower oil-pyridoxine-lipotropic product was 0.461. At the 18 week mark that ratio was 0.580, representing a difference of 0.120. The standard deviation of difference was 0.102; standard error of the mean difference was

0.022. Thus, the mean difference divided by the standard mean difference was 5.4, which is highly significant. The odds against occurrence of a deviation as great as, or greater than, 0.12 (increase in the alpha to beta lipoprotein ratio after 18 weeks) are approximately 2,000,000 to 1.

Shortly after the 18 week mark, patient F.E. (No. 22), 38-year-old white male, suddenly died of acute myocardial infarction while undergoing extreme physical and emotional stress. Autopsy revealed marked and widespread coronary atherosclerosis. It is of interest that this patient's initial alpha to beta ratio was 0.35 and 18 weeks after safflower oil administration it rose to 0.42. His total serum cholesterol had fallen from an initial 592 mg% to 525 mg% at 18 months.

Six out of 25 patients showed decreased total serum cholesterol levels at the 18 or 30 week period.

Discussion. Major aberration in lipid metabolism as evidenced by greatly elevated total serum cholesterol, elevated fasting chylomicron index or even frank lipemia are predominant factors in premature and severe coronary atheromatosis(7,8,9). The difficulty lies in the borderline cases which comprise the majority of our clinical material, where one can apply only group correlation.

Paper electrophoresis is a reliable quantitative method for measurement of alpha and beta lipoprotein(10). Determination of the ratio of alpha to beta lipoprotein in fasting serum is in our experience the most sensitive index of the patient's individual ability to metabolize fat.

Since ingestion of large amounts of medication is disagreeable to many patients, we used moderate doses and the present report deals with the results obtained with those doses. Kinsell *et al.*(11) reported that the 24-hour intake of 2 g of linoleic acid resulted in prompt and profound decrease in plasma cholesterol and other lipid fractions.

Moderate doses of safflower seed oil resulted in reduction of serum cholesterol in 6 patients. A statistically significant shift from beta to alpha lipoprotein occurred in a large percentage of our patients after 18 weeks of

[†] The combination of safflower seed oil, pyridoxine and lipotropics were administered in capsule form, coded LUFA.

TABLE II. Effect of Safflower Seed Oil - Pyridoxine - Lipotropic Medication.

| Patient | Age | Sex | Before medication | | 12 wk | | 18 wk | | 24 wk | | 30 wk | |
|---------|-----|-----|----------------------|-------------------|----------------------|-------------------|----------------------|-------------------|----------------------|-------------------|----------------------|-------------------|
| | | | α/β ratio | Cholesterol, mg % | α/β ratio | Cholesterol, mg % | α/β ratio | Cholesterol, mg % | α/β ratio | Cholesterol, mg % | α/β ratio | Cholesterol, mg % |
| 1 | 52 | ♂ | .25 | 328 | .51 | 348 | .42 | 321 | .30 | 296 | .50 | 277 |
| 2 | 49 | ♂ | .39 | 295 | .46 | 340 | .65 | 374 | .52 | 343 | .59 | 349 |
| 3 | 67 | ♂ | .51 | 207 | .72 | 220 | | | | | .75 | 210 |
| 4 | 68 | ♂ | .30 | 409 | .43 | 396 | .40 | 408 | .44 | 395 | .28 | 378 |
| 5 | 63 | ♂ | .39 | 332 | .35 | 319 | .43 | 326 | .39 | 313 | .41 | 303 |
| 6 | 56 | ♂ | .41 | 262 | .45 | 272 | .42 | 286 | .62 | 287 | .49 | 286 |
| 7 | 55 | ♂ | .43 | 289 | .58 | 303 | .57 | 302 | .47 | 305 | .51 | 297 |
| 8 | 71 | ♀ | .36 | 320 | | | .58 | 316 | .32 | 369 | .39 | 306 |
| 9 | 61 | ♀ | .37 | 429 | | | .38 | 508 | .47 | 517 | .32 | 485 |
| 10 | 54 | ♂ | .27 | 220 | .44 | 264 | .51 | 260 | .40 | 241 | .51 | 216 |
| 11 | 63 | ♂ | .30 | 248 | .55 | 264 | .68 | 272 | .49 | 241 | .63 | 228 |
| 12 | 51 | ♀ | .44 | 311 | .42 | 325 | .42 | 334 | .44 | 257 | .42 | 326 |
| 13 | 51 | ♂ | .74 | 251 | | | .93 | 248 | | | .98 | 261 |
| 14 | 43 | ♀ | .59 | 280 | .62 | 260 | .67 | 326 | | | .75 | 257 |
| 15 | 78 | ♂ | .55 | 198 | .43 | 200 | .71 | 178 | | | .60 | 188 |
| 16 | 72 | ♀ | .40 | 362 | | | | | | | .61 | 326 |
| 17 | 59 | ♂ | .71 | 284 | .52 | 294 | .72 | 290 | .59 | 300 | .69 | 248 |
| 18 | 62 | ♂ | .43 | 330 | .39 | 272 | | | .56 | 290 | .67 | 248 |
| 19 | 58 | ♀ | .80 | 291 | .60 | 295 | .80 | 290 | .63 | 284 | .65 | 289 |
| 20 | 51 | ♀ | .61 | 336 | .64 | 216 | .94 | 358 | .64 | 314 | .79 | 316 |
| 21 | 42 | ♂ | .42 | 265 | .52 | 272 | .50 | 264 | .46 | 282 | .58 | 253 |
| 22 | 36 | ♂ | .35 | 592 | .33 | 524 | .42 | 525 | | | | |
| 23 | 51 | ♀ | .61 | 250 | .87 | 269 | .67 | 272 | 1.04 | 296 | .74 | 261 |
| 24 | 63 | ♂ | .39 | 277 | .39 | 248 | .48 | 267 | .58 | 260 | .44 | 224 |
| 25 | 40 | ♀ | .49 | 279 | | | .85 | 238 | | | | |

treatment. The alpha to beta ratio continued to rise at 24 or 30 weeks of medication in 18 cases out of 24. This consistent trend encourages us to proceed with the study over a longer period of time and with larger doses of safflower seed oil. Our studies indicate that essential fatty acids do exert an unequivocal effect on serum lipids; in larger quantities they apparently lower total serum cholesterol. In moderate doses, which we consider practical from the patient standpoint, there is a consistent and permanent shift from beta to alpha lipoprotein.

Summary. 1. The present study confirmed the group correlation between clinically demonstrable coronary atherosclerosis and abnormally low alpha to beta lipoprotein ratio. 2. Daily administration of 3 g of safflower seed oil and 4 mg of pyridoxine for a period of up to 30 weeks, added to the previously administered lipotropes, resulted in a statistically significant increase of alpha to beta lipoprotein ratio starting at the 18 week period. The trend continued for the 24 and 30 week periods in over 70% of cases. 3. Twenty per

cent of cases showed lowered total serum cholesterol levels. 4. In view of the potential significance of the observed results, further study is merited.

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Tentative Pattern for Renewal of Lymphocytes in Cortex of the Rat Thymus.* (23710)

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It is not known how the 4 cell types present in the thymus (reticular cells, large, medium and small lymphocytes) are involved in lymphocyte production(1-3). In the hope of clarifying the problem, the numbers of these cells and their mitoses were counted in different regions of the organ, and an attempt made to elaborate a pattern of lymphocyte renewal consistent with the experimental data. The present article is a preliminary report on the results and conclusions reached so far.

Methods. Twenty-eight 10-week-old male albino rats were divided into 4 groups of 7 animals each, sacrificed at 10 a.m., 4 p.m. and 10 p.m. during same day, and at 4 a.m. the next morning respectively. After fixation for 2 days in modified Bouin-Hollande (4 g picric acid; 2.5 g copper sulfate; 20 ml neutral formalin; 0.75 ml acetic acid; 0.75 g trichloroacetic acid and 100 ml water), the tissues were sectioned at 5 μ and stained by the Dominici technic.

Results. Cytological criteria (Fig. 1). The *reticular cell* has a pale cytoplasm which is little or not visible and a large nucleus (to 11 μ diameter), oval or diamond-shaped, which appears very light. Fine chromatin dots are associated with the nuclear membrane. There is usually one, and sometimes 2-4 large oval nucleoli, the center staining orange-red, the surface light blue. A loose network of fine chromatin threads connects the nucleolus to nuclear membrane.

The *large lymphocyte* has a basophilic, well-limited cytoplasm, arranged into a more or less regular ring around the nucleus. The nucleus is usually round, diameter equal to or greater than 5.9 μ and stains slightly darker than in the reticular cell. Larger chromatin dots than in the reticular cell are associated with the nuclear membrane. There are 1-4 nucleoli of variable shape (oval, stellate, elongated) with an acidophilic core and a more bluish chromatin coat than in the reticular cell. The thread network within the nucleus is slightly denser than in the reticular cell.

The *medium lymphocyte* usually has a small ridge of basophilic cytoplasm, less in amount than in the large lymphocyte. The nucleus which measures 4.6-5.9 μ is often round and the nuclear sap appears blue with a purplish hue. Coarser chromatin masses than in the large lymphocyte are associated

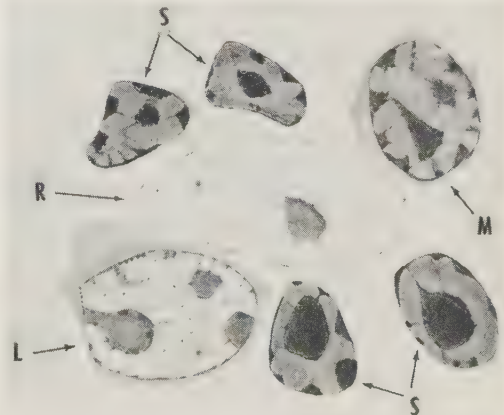


FIG. 1. Drawing of cells of thymus: R, reticular cell; L, large lymphocyte; M, medium lymphocyte; S, small lymphocyte.

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† Fellow of the Nat. Cancer Inst. of Canada.

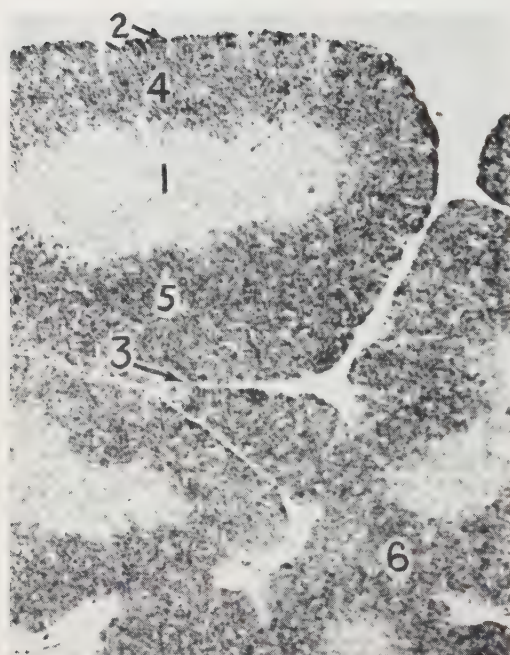


FIG. 2. Microphotograph of a portion of thymus from 10-wk-old male rat showing the 6 zones in which resting and dividing nuclei were enumerated.

with the nuclear membrane. The 1-3 nucleoli are smaller, more regular and have a darker blue coat than in the large lymphocyte.

The *small lymphocyte* has cytoplasm so scanty as not to be visible in most cases. The shape of nucleus is more variable than in the other 2 types of lymphocyte, the diameter is less than 4.6μ . Many large masses of chromatin are associated with the nuclear membrane. The nucleolus is located towards the center of the cell and measures up to 1.7μ diameter. It is composed of an acidophilic core and a chromatin coat which appears darker than in any other cell type.

Owing to the small amount of cytoplasm present in lymphocytes, the diameter of mitotic figures is approximately the same as that of resting nuclei. Hence, dimensions given above were used for identification of the mitotic figures of the various cell types.

Cell counts. Resting and dividing cells were counted at magnification of about 1000x using square microscopic fields ($25 \times 25 \mu$) in 6 different zones of the thymus (Fig. 2): 1) medulla, which will not be considered here; 2) subcapsular peripheral cortex (within 25μ

of capsule); 3) subtrabecular peripheral cortex (within 25μ of trabeculae); 4) subcapsular deep cortex; 5) subtrabecular deep cortex; and 6) intermedullary deep cortex. Resting cells were counted in the first 2 groups of animals but mitoses, including early prophase and late telophases, in all 4.

In each group, zones 2 and 3 yielded approximately the same counts/field for each cell type. Counts from these 2 zones were pooled and recorded for each animal under heading "peripheral cortex." For the same reason, results for zones 4, 5 and 6 were pooled and recorded as "deep cortex." Since the peripheral cortex makes up 9% and the deep cortex 66% of the parenchymal thymus, weighted averages were calculated for each count as follows:

$$\frac{(\text{peripheral cortex count} \times 9) + (\text{deep cortex count} \times 66)}{75}$$

and recorded under the heading "total cortex."

In either the peripheral or deep cortex, the number of cells/field did not vary over a 24-hour period, but mitotic activity showed slight, significant variations. We averaged the figures for all animals available and thus obtained values representative of a whole day. The mean numbers of cells and mitoses/field reported in Table I with their standard errors were thus obtained. In addition, the mitotic index for each cell type, that is, the ratio of number of mitoses over the sum of the numbers of resting cells and mitoses was calculated (Table I).

Basic assumptions. The assumptions which will be used in the discussion, together with a brief statement of the supporting evidence, are listed below:

1) *Four cell types are present.* The separation of cells into 4 types was justified by the fact that, using cytological criteria detailed above, reproducible counts of cells and their mitoses could be obtained.

2) *The 4 cell types are being continuously renewed.* This assumption is upheld since the mitotic indices in the 4 cell types (Table I) greatly exceeded mitotic indices observed in cell populations which are not renewed (under 0.002; see the discussion in ref. 4). Renewal implied that cells of the 4 types di-

TABLE I. Number of Cells and of Mitoses per Field and Mitotic Index in Peripheral, Deep, and Total Cortex of Thymus of 10-Wk-Old Rats.

| | Peripheral cortex | | | Deep cortex | | |
|-------------------|-------------------|----------------|---------------|--------------|----------------|---------------|
| | No. of cells | No. of mitoses | Mitotic index | No. of cells | No. of mitoses | Mitotic index |
| Reticular cells | .28 ± .06† | .005 ± .002 | .016 | .19 ± .03 | .003 ± .001 | .015 |
| Large lymphocytes | 1.07 ± .11 | .079 ± .012 | .069 | .60 ± .08 | .041 ± .004 | .065 |
| Medium " | 1.92 ± .12 | .295 ± .020 | .133 | 1.04 ± .05 | .156 ± .011 | .130 |
| Small " | 14.3 ± .45 | .379 ± .025 | .026 | 16.0 ± .39 | .221 ± .011 | .014 |
| Total cortex* | | | | | | |
| Reticular cells | .20 ± .03 | .003 ± .001 | .015 | | | |
| Large lymphocytes | .66 ± .08 | .046 ± .004 | .065 | | | |
| Medium " | 1.15 ± .05 | .172 ± .011 | .131 | | | |
| Small " | 15.75 ± .39 | .240 ± .011 | .015 | | | |

* Weighted averages for total cortex based on relative volumes of peripheral and deep cortex.

† ± S.E. in all cases.

vided at frequent intervals to reproduce themselves and/or produce cells of another type.

3) *Small lymphocytes arise from medium, which come from large lymphocytes, which in turn come from reticular cells.* This assumption, which has been more or less clearly adopted by many investigators of the lymphocyte(1,5-7), was supported by existence of many transition forms. The progressive increase in numbers of cells and of mitoses from reticular cells to small lymphocytes (Table I) was also in agreement with this assumption.

4) *The life span (intermitotic time + mitotic duration) is the same for all cells of a given type.* Since the more frequently cells enter mitosis, the shorter their life span is, it is clear that the mean life span is inversely proportional to the mean mitotic index. Hence, the present assumption requires constancy of the mitotic index for each cell type. This appeared to be the case for reticular cells, large and medium lymphocytes, since they had approximately the same mitotic index in both peripheral and deep cortex (Table I). (Complicating factors with regard to small lymphocytes will be mentioned below.) It must not be forgotten that the existence of diurnal variations in mitotic activity indicates inverse fluctuations in life span and, therefore, the life span under consideration is a mean figure for the 24-hour period. However, it is likely that, even at a given time, the cells of one type have the same life span, since islands formed of similar cells which occasionally undergo mitosis simultaneously were commonly seen in sections.

5) *Mitotic duration is taken to be the same in all cells of the thymus.* This assumption is justified by the results of Widner *et al.*(8), who showed that mitotic duration was approximately the same for a variety of cells in the rat.

6) *Only small lymphocytes leave the thymus in significant numbers,* an assumption based on the predominance of small lymphocytes in lymph and blood vessels.

Further assumptions, which concern the life span of small lymphocytes but do not affect our main argument, will be mentioned later.

Discussion. It has been shown that, when nuclei are counted in sections, the count is higher than the actual number of nuclei(9). Errors are directly related to nuclear size (9), and tend to cancel out when ratios of nuclei of similar size are considered. Accordingly, the data in Table I were used to calculate such ratios for cells with nuclei fairly similar in size. When the ratios of large lymphocytes to reticular cells, and of medium to large lymphocytes were calculated, they were approximately the same for peripheral, deep, and total cortex; therefore, only those ratios obtained for total cortex are reported in Table II.†

However, the ratio of small to medium

† The formula used to calculate the standard errors of the ratios was of the form $\pm \frac{1}{B^2} \sqrt{B^2 a^2 + A^2 b^2}$ in which A and B are respectively the numerator and denominator of the ratio, and a and b their respective standard errors.

TABLE II. Experimental Ratios of Number of Cells, Mitoses and of Mitotic Indices for Total Cortex of Thymus of 10-Wk-Old Rats.

| Cells involved in ratios | Ratios of | | |
|--------------------------|-----------------------|----------------|---------------|
| | No. of cells | No. of mitoses | Mitotic index |
| Large lymphocytes | $3.3 \pm .58^\dagger$ | 14.8 ± 3.5 | 4.3 ± 1.2 |
| Reticular cells | | | |
| Medium lymphocytes | $1.8 \pm .22$ | $3.8 \pm .38$ | $2.0 \pm .31$ |
| Large lymphocytes | | | |
| Small lymphocytes* | $7.4 \pm .53$ | $1.3 \pm .12$ | $.19 \pm .02$ |
| Medium lymphocytes | | | |

* In the case of small lymphocytes, only the figures obtained in the peripheral zone of the cortex were used.
† \pm S.E.

lymphocytes in deep cortex (15.3) was about twice that in peripheral cortex (7.4), a fact suggesting that some accumulation of small lymphocytes takes place in deep cortex (see end of section on "Relation of small to medium lymphocytes"). It was therefore decided that the counts of small lymphocytes obtained in the peripheral cortex would be used in calculating the ratios in Table II.[‡]

The purpose of the present discussion is to find a pattern for lymphocyte renewal consistent with the 9 ratios in Table II.

Relation of medium to large lymphocytes. Since the ratio of mitotic indices of medium to large lymphocytes is 2 (Table II), it follows that the life span of medium must be half that of large lymphocytes. It was decided to calculate on this basis what the ratios of the mean numbers of cells and of mitoses of these 2 cell types would be if one generation of medium lymphocytes were to follow either one, 2 or more generations of large lymphocytes. Let us first consider the simplest possibility, that is, single generations of large (L_1) and medium (M_1) lymphocytes, as represented in diagram I of Fig. 3 (in which the vertical dimension is time). Any large lymphocyte would then divide to give rise to 2 medium lymphocytes, which would in turn divide to produce small lymphocytes. Thus, medium lymphocytes would be twice as numerous as large lymphocytes, but since they live only half as long, both cell types would be found in sections in equal numbers and the ratio of the total number of medium to large lymphocytes would be 1 (Table III). It is

evident that this ratio is smaller than that recorded in the results in Table II, so that the possibility presented in diagram I may be discarded. If 2, 3 or more generations of large lymphocytes preceded one of medium lymphocytes, similar reasoning would indicate that in each case the ratio of the numbers of cells (Table III) would be much smaller than that obtained experimentally. Accordingly,

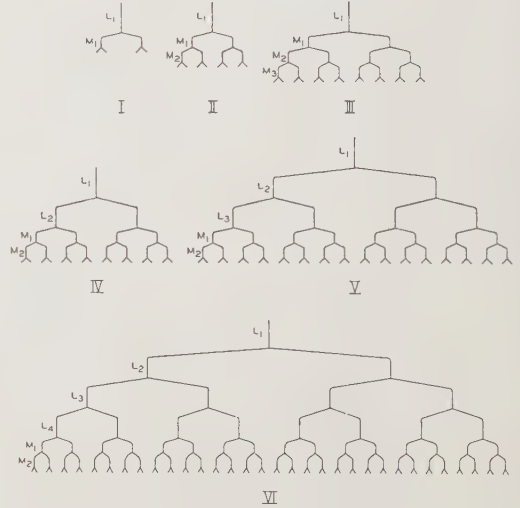


FIG. 3. Diagrammatic representation of possible patterns for the relation of large to medium lymphocytes (1 generation of large to 1, 2 or 3 generations of medium lymphocytes in diagrams I, II and III respectively; 2, 3 or 4 generations of large to 2 generations of medium lymphocytes in diagrams IV, V and VI respectively). The letters L, M and S refer to large, medium and small lymphocytes and the subscript numbers to the cell generation involved. The mitoses are indicated on the diagrams by the junction of a vertical and 2 oblique lines. (Vertical dimension in this and following diagram indicates time.)

TABLE III. Expected Ratios for Number of Medium to That of Large Lymphocytes and for Number of Mitoses of These 2 Cell Types, According to Number of Cell Generations.

| Relative No. of cell generations | | Diagram No. (Fig. 3) | Ratios of | |
|----------------------------------|-------------------|----------------------|--------------|----------------|
| Medium lymphocytes | Large lymphocytes | | No. of cells | No. of mitoses |
| 1 | 1 | I | 1.00 | 2.00 |
| | 2 | | .66 | 1.33 |
| | 3 | | .57 | 1.14 |
| | 4 | | .53 | 1.06 |
| | 5 | | .52 | 1.04 |
| 2 | 1 | II | 3.0 | 6.0 |
| | 2 | IV | 2.0 | 4.0 |
| | 3 | V | 1.7 | 3.4 |
| | 4 | VI | 1.6 | 3.2 |
| | 5 | | 1.5 | 3.1 |
| 3 | 1 | III | 7.0 | 14.0 |
| | 2 | | 4.7 | 9.3 |
| | 3 | | 4.0 | 8.0 |
| | 4 | | 3.7 | 7.4 |
| | 5 | | 3.6 | 7.2 |

In all cases, the mitotic index of medium is twice that of large lymphocytes.

it was concluded that there must be more than one generation of medium lymphocytes.

On the basis of 2 generations of medium lymphocytes following one generation of large lymphocytes (Diagram II, Fig. 3), there would be 1 large lymphocyte (L_1) for either 2 or 4, that is, a mean of 3 medium lymphocytes (M_1, M_2), so that the ratio of the number of medium to large lymphocytes would be 3. Furthermore, the odds of finding medium lymphocytes undergoing mitosis would be 6 times as great as those of finding a large lymphocyte undergoing mitosis, so that ratios of numbers of mitoses would be 6 (Table III). Neither the ratio of the number of cells nor that of mitoses agreed with the experimental findings (Table II). Hence, the possibility illustrated in diagram II was discarded. Calculations were then made on the basis of 2 generations of medium following either 2 (diagram IV), 3 (diagram V), 4 (diagram VI) or 5 generations of large lymphocytes. The data (Table III) revealed that a fair agreement with the experimental results was obtained in each one of the 4 cases. It thus appeared that there were 2 generations of medium lymphocytes, but the number of preceding generations of large lymphocytes remained to be established.

Relation of large lymphocytes to reticular cells. Calculations were then made for 2 to 5 generations of large lymphocytes combined with a variable number of generations of reticular cells. Examination of the ratios of numbers of mitoses (Table IV) indicated that a figure approaching the experimental number (14.8) was obtained only with 4 generations of large lymphocytes. Examination of ratios of numbers of cells (Table IV) revealed that, with 4 generations of large lymphocytes, one generation of reticular cells provided the best fit with the experimental data. However, other possibilities (e.g. 0.8 generation of reticular cells for 4 of large lymphocytes) yield ratios which are also fairly close to the experimental figures and cannot be definitely discarded. (The standard errors for number of mitoses of reticular cells were high, Table I. A recount of the mitoses of these cells in 5000 fields gave figures within the range of the errors. Even so, conclusions relating to reticular cells may be considered to be more tentative than those relating to other cell types).

Relation of small to medium lymphocytes. The next step was to investigate the number of generations of small lymphocytes which

TABLE IV. Expected Ratios for Number of Large Lymphocytes to That of Reticular Cells and for Number of Mitoses and Mitotic Indices of These 2 Cell Types According to the Number of Cell Generations.

| Relative No. of cell generations | | Ratios of | | |
|----------------------------------|-----------------|--------------|----------------|---------------|
| Large lymphocytes | Reticular cells | No. of cells | No. of mitoses | Mitotic index |
| 2 | .5 | .7 | 3 | 4 |
| | 1 | 1.5 | 3 | 2 |
| | 2 | 3.0 | 3 | 1 |
| 3 | .5 | 1.2 | 7 | 6 |
| | .75 | 1.7 | 7 | 4 |
| | 1 | 2.3 | 7 | 3 |
| | 1.20 | 2.8 | 7 | 2 |
| | 1.5 | 3.5 | 7 | 2 |
| | 3 | 7.0 | 7 | 1 |
| 4 | .80 | 3.0 | 15 | 5 |
| | 1 | 3.7 | 15 | 4 |
| | 1.33 | 5.0 | 15 | 3 |
| | 2 | 7.5 | 15 | 2 |
| | 4 | 15.0 | 15 | 1 |
| 5 | 1 | 6.2 | 31 | 5 |
| | 2.5 | 12.4 | 31 | 2 |
| | 5 | 31.0 | 31 | 1 |

TABLE VI. Comparison of Experimental Ratios of Number of Cells, Mitoses and of Mitotic Indices with Ratios Expected on Basis of Diagram in Fig. 4.

| Cells involved in ratios | No. of cells | | No. of mitoses | | Mitotic index | |
|--------------------------|--------------|----------|----------------|----------|---------------|----------|
| | Exp. | Expected | Exp. | Expected | Exp. | Expected |
| Large lymphocytes | | | | | | |
| Reticular cells | 3.3 ± .58† | 3.7 | 14.8 ± 3.5 | 15.0 | 4.3 ± 1.2 | 4.0 |
| Medium lymphocytes | | | | | | |
| Large lymphocytes | 1.8 ± .22 | 1.6 | 3.8 ± .38 | 3.2 | 2.0 ± .31 | 2.0 |
| Small lymphocytes* | | | | | | |
| Medium lymphocytes | 7.4 ± .53 | 7.3 | 1.3 ± .12 | 1.3 | .19 ± .02 | .18 |

* In the case of small lymphocytes, only the figures obtained in the peripheral zone of the cortex were used.

† ± S.E. in all cases.

goes division. Meanwhile, each 4th generation large lymphocyte yields medium lymphocytes, which go rapidly through 2 generations to produce small lymphocytes, which in turn divide to yield a total of 128 mature small lymphocytes. Examination of cells associated with the reticular cell at any given time makes it possible to subdivide the sequence of events into 4 stages (referred to as stages I, II, III and IV in Fig. 4), which follow one another indefinitely. Thus each reticular cell and its progeny would be going through a self-repeating cycle composed of these 4 stages. This observation yields a simple method of calculating the expected ratios of the cells. The number of cells and the odds of occurrence of mitosis were determined at each stage and averaged (Fig. 4). The ratios of the means are reported in Table VI as the "expected" values. It may be seen in Table VI that the expected values fall within the range of standard errors of the experimental figures in all cases (except for the ratio of number of mitoses of medium to that of large lymphocytes, which is on the borderline). It is concluded that the agreement between experimental and expected ratios is close; and, therefore, the scheme appears to be statistically valid. However, eventual refinement of the technic or variations in the assumptions may yield a similar or slightly different scheme which also fits the data. The pattern is considered to be sufficiently close to the observed facts to be used as a tool in future experimental work. It is proposed to refer to this scheme as the "Stem cell renewal theory" of lymphocyte formation in the thymus.

mus.

Summary. 1) Counts of resting and dividing cells in the cortex of the thymus of 10-week-old male rats reveal a high mitotic index of the 4 cell types present: reticular cells, large, medium and small lymphocytes. This observation indicates that each one of the 4 cell types is being renewed. Presumably this renewal yields lymphocytes which leave the thymus and enter the circulation. 2) From the mean numbers of cells and of mitoses and the mean mitotic indices of the four cell types over a 24-hour period, a tentative scheme referred to as "Stem cell renewal theory" is presented to account for the continuous production of lymphocytes in the thymus. This theory (Fig. 4) is that each reticular cell at regular intervals yields large lymphocytes which pass through 4 generations and then produce medium lymphocytes, which in turn pass through 2 short-lived generations and then give rise to small lymphocytes, of which there are also 2 successive generations.

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Long Term Endogenous Creatinine Clearance in Man.* (23711)

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Endogenous creatinine clearances are similar to inulin clearances in the normal individual when run *simultaneously* for short periods of time (5-15 minutes)(1-3) and also when run *independently* (no inulin infusion) for long periods of time (1-24 hours)(4-5). Factors that might influence the endogenous creatinine clearance were investigated. Creatinine clearances were determined on 39 normal individuals and the values compared with reported short term inulin clearance values for individuals of the same age group—the determination of inulin clearances on the group of volunteer subjects was not possible.

Methods. All subjects were free from hypertension, muscle or kidney disease. Urine was collected without catheterization, a 1:1000 dilution made and 5 ml removed for analysis. Blood was obtained by venipuncture and placed in a tube containing potassium oxalate. Protein was removed from the plasma by the sodium tungstate-sulfuric acid method of Folin-Wu(6). Creatinine was determined by the method of Van Pilsum *et al.* (7).† Urea was determined by the Van Slyke(8) modification of the Folin-Wu(6) method.

Results. The effect of urinary volume variations on simultaneous creatinine and urea clearances was determined on 2 normal adult males. Clearances were of one or 2 hours duration and were run consecutively. Blood

was withdrawn at the beginning and the end of each clearance period and the average of the 2 values was used in the calculation of the clearance. Urea clearance was calculated as standard clearance (C_s) when the rate of flow was less than 2 ml per minute; when greater than 2 ml per minute, it was calculated as maximum clearance (C_m). Creatinine clearance was calculated according to the clearance

formula $\frac{UV}{P}$. Immediately preceding and during the clearance period, the subjects assumed normal activities, but avoided vigorous exercise or the ingestion of large amounts of meat. Diuresis was produced by ingestion of water. The results are shown in Table I. The range of variation of 139 to 169 ml per minute (subject 1) and of 123 to 139 ml per minute (subject 2) is of the same order of

TABLE I. Effects of Urinary Volume Variations on Simultaneous Creatinine and Urea Clearances in the Normal Adult Male.

| Urine vol (ml/min.) | Creatinine clearance (ml/min./1.73 m ²) | Urea clearance (ml/min./1.73 m ²) | |
|------------------------|---|--|----------------|
| | | C _s | C _m |
| 1. | .88 | 169 | 63 |
| | 1.08 | 139 | 72 |
| | 3.00 | 161 | 115 |
| | 5.00 | 167 | 151 |
| | 3.00 | 161 | 94 |
| | 1.53 | 152 | 74* |
| 2. | 1.40 | 139 | 59 |
| | 1.22 | 123 | 58 |
| | 1.45 | 126 | 60 |
| | 2.05 | 137 | 80 |
| | 9.95 | 131 | 114 |

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† In this method creatinine is degraded to methylguanidine by O-nitrobenzaldehyde in the presence of alkali. The methylguanidine is measured with a modified Sakaguchi color reaction.

Standard clearance (C_s) = $\frac{UV\sqrt{V}}{P}$; maximum clearance (C_m) = $\frac{UV}{P}$ (10).

* Clearances calculated according to the formula $\frac{UV}{P}$, regardless of rate of urine flow.

magnitude reported in consecutive inulin clearances(2-3). The urea clearances showed a greater range of variation, as has been previously reported(9).

The effects of protein ingestion, exercise, and length of clearance are shown in Table II. For the protein ingestion experiment the subjects fasted 12 hours and then a 3 hour clearance run. The subjects ingested large amounts of well cooked protein (approximately 300 g) and a 3 hour clearance run. Blood was withdrawn at the middle of each clearance period. The exercise experiment was determined similarly, except a period of $\frac{1}{2}$ hour of vigorous exercise was substituted for protein ingestion. In the length of clearance experiment the one and 24 hour clearances were done consecutively, with blood samples withdrawn at the end of each clearance period, the 24 hour plasma creatinine value being the average of the two samples. Both exercise and a high protein meal seemed to increase the clearance a small amount. The 1 hour and 24 hour clearances were similar.

Creatinine clearances were determined on 39 individuals of both sexes varying in age from 3-99 years and the results compared with reported inulin clearances for the same age groups (Table III). All clearances were of

TABLE III. Normal Endogenous Creatinine Clearances.

| Age group | No. of subjects | Reported inulin clearance | |
|-----------|-----------------|---------------------------|--------------------------------|
| | | Clearance | (ml/min./1.73 m ²) |
| 3-20 | 6 | 127 \pm 16 | *129 \pm 17 (2) |
| 20-50 ♂ | 11 | 135 \pm 14 | 131 \pm 21(11) |
| 20-50 ♀ | 7 | 127 \pm 18 | 117 \pm 15(11) |
| 60-69 | 2 | 105 \pm 7 | 96 \pm 25(12) |
| 70-79 | 3 | 104 \pm 16 | 89 \pm 20(12) |
| 80-89 | 7 | 74 \pm 22 | 65 \pm 20(12) |
| 90-99 | 3 | 38 \pm 8 | |

All values are avg \pm stand. dev.

* Calculated from data reported for age group 4-12.5.

at least 8 hours duration, the majority 24 hours. The plasma creatinine level was determined from a single sample at the middle of the clearance period or by the average of samples withdrawn at the beginning and at the end of the clearance period. In the age groups from 3-20 and 20-50 years there was good agreement between long term creatinine clearances and reported short term inulin clearances for these same age groups. In the age groups from 60-99 years there was a gradual consistent lowering of creatinine clearances, parallel with increase in age, which also agreed well with reported inulin clearances.

Summary. The effects of urinary volume, diet, and exercise on long term endogenous creatinine clearance were studied on normal individuals using the ortho-nitrobenzaldehyde method for creatinine. Urinary volume variations had no effect on creatinine clearance; protein ingestion and exercise seemed to increase slightly the clearance values. One hour clearances were similar to 24 hour clearances. Long term creatinine clearances of 39 normal individuals of varying age groups were determined and were found to be similar to reported inulin clearance values of these same age groups.

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TABLE II. Effects of Protein Ingestion, Exercise, and Length of Clearance on Creatinine Clearance.

| Subject No. | Clearance | |
|-------------|--------------------------------|----------------|
| | (ml/min./1.73 m ²) | |
| | Fasting | High protein |
| 1 | 152 | 177 |
| 2 | 119 | 147 |
| 3 | 122 | 145 |
| 4 | 135 | 138 |
| Avg | 132 | 152 |
| | Before exercise | After exercise |
| 1 | 146 | 158 |
| 2 | 129 | 143 |
| 3 | 125 | 140 |
| 4 | 169 | 180 |
| Avg | 142 | 155 |
| | 1 hr | 24 hr |
| 5 | 139 | 141 |
| 6 | 163 | 150 |
| 7 | 137 | 150 |
| 8 | 147 | 167 |
| 9 | 175 | 160 |
| 10 | 150 | 152 |
| 11 | 118 | 123 |
| Avg | 147 | 149 |

urine samples. We would like to express our gratitude to N. E. Quam of the Ebenezer Home and T. Bruich for help in obtaining blood and urine samples in the aged.

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Influence of Environmental and Skin Temperature on Threshold for Nicotine Axone Reflex Sweating in Humans.*† (23712)

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The major characteristics of axone reflex sweating and piloerection, elicited by intradermally injected nicotinic drugs in the human, have been elucidated through the efforts of several workers(1,2). As yet no evidence exists either that these axone reflexes occur physiologically or, if they do, what role they might play. One possibility is that such a peripheral mechanism might serve to modify the response of the cutaneous effectors to their central nervous controls. Determination of effects of environmental and skin temperature changes on the characteristics of nicotine sweating appeared to offer an indirect test of this hypothesis. Measurement of threshold effects proved simpler and probably more accurate than measurement of changes in ex-

tent of suprathreshold responses, although some of the latter were essayed. The present study was restricted chiefly to the sweat response, although some corollary data on piloerection are also given. A very closely related investigation is that of Benjamin(3) who, studying the response of sweat glands to locally applied heat, showed an interaction between this stimulus on the one hand, and environmental temperature and locally injected acetylcholine on the other.

Methods. Healthy, white individuals, aged 20 to 34, were used as subjects; all but one experiment, noted below, were done on males. Injections of nicotine were made intradermally on the volar forearm with 26 gauge needles and 1 or 1/2 ml tuberculin syringes; in all cases, the volume injected was 0.05 ml. Solutions were made by diluting nicotine alkaloid to appropriate concentrations in sterile 0.9% NaCl; concentrations are expressed in terms of weight to volume. Sweating was detected by Randall's(4) iodine-starch paper method; the response was quantitated in terms of area and intensity on arbitrary scales. Piloerection was noted visually and, because of difficulty in quantization, noted only as

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TABLE I. Effect of Local Heating on Nicotine Sweat Threshold.

| Subject 1 | | | | Subject 2 | | | | |
|-----------------|---------------------|-----------------|---------------------|-----------------|-----------------|--------------------|-----------------|--------------------|
| —Left arm— | | —Right arm* | | Room temp. (°C) | —Left arm* | | —Right arm— | |
| Skin temp. (°C) | Nicotine threshold | Skin temp. (°C) | Nicotine threshold | | Skin temp. (°C) | Nicotine threshold | Skin temp. (°C) | Nicotine threshold |
| | | 34.5 | $1:1.6 \times 10^6$ | 26 | 34 | $1:1 \times 10^6$ | | |
| | | 31.0 | $1:1.2 \times 10^6$ | 16 | 29 | $>1:6 \times 10^5$ | | |
| | | | | Heat on arm | | | | |
| | | | | 16 | 38 | $1:1 \times 10^6$ | 29 | $>1:6 \times 10^5$ |
| 32.0 | $1:1.2 \times 10^6$ | 36.5 | $1:1.4 \times 10^6$ | Heat off arm | | | | |
| | | | | 16 | 29 | $>1:6 \times 10^5$ | | |
| | | 29.0 | $1:1 \times 10^6$ | | | | | |

* Heated arm.

present or absent.

Results. The threshold concentration of intradermal nicotine for producing the sweat response at room temperature (23° – 26°C) varied from 1:800,000 to 1:2,000,000 among the individuals tested. Considerable variation was noted from day to day for any one subject. In 4 subjects, the magnitude of sweating was determined at a range of concentration from 1:50,000 to 1:1,000,000. Maximal sweating was observed between 1:400,000 and 1:800,000, with gradual diminution at increased and decreased concentrations outside this range. In the same subjects, axone reflex piloerection was observed at concentrations of 1:200,000 to 1:1,000,000, the maximum response appearing to coincide with the maximum sweating response.

The effects on threshold of altering the environmental temperature were studied on four males and one female. In each case the nicotine threshold was first determined at room temperature (26° – 27°C); the subject was then placed in a room at 15° (in one case, 18°) for at least 30 minutes and retested; the subject was then returned to the previous environment for at least 30 minutes and threshold again determined. Skin temperature was monitored with a thermocouple or a mercury thermometer; the indicated temperatures of the 2 devices corresponded within 1°C . At each environmental temperature, skin temperature was allowed to stabilize before tests were made. In all subjects the threshold at 15° or 18° was at least 15% higher than it had been at 26° ; on return to 26° , the threshold fell towards its original level. In each case, skin temperature fell from 34° – 35°

(room temperature of 26°) to 29° – 32.5° (room temperature of 15° – 18°).

To evaluate the role of local skin temperature in the sweating response to nicotine, a modification of the above experiment was performed in 2 subjects (Table I). The threshold was determined first on one forearm at room temperature. The subject was put in the cold room (16°) until skin temperature stabilized, whereupon threshold was again determined on that arm. This arm was then heated by an infra-red lamp until skin temperature reached 36.5° – 38° , and nicotine threshold again determined on the heated arm, and on the opposite, unheated, arm as well. The heat lamp was then turned off and threshold retested on the previously heated arm after skin temperature had fallen again to 29° . As indicated in Table I, elevation of local temperature was adequate to cause a fall in nicotine threshold.

Discussion. Two major conclusions follow from these observations. 1) The dose-response characteristics of nicotine in producing axone reflex sweating are similar to those of its "nicotinic" action at autonomic ganglia: above a certain optimal concentration the response is self-inhibitory. This confirms the views of Coon and Rothman(1). 2) The threshold for nicotine sweating is a function of ambient temperature. This relationship is due in large part, if not entirely, to the effect of alterations in local skin temperature on sensitivity of the cutaneous components of the axone reflex mechanism. This is shown by the significant change wrought by isolated alteration of skin temperature. This result is parallel to Benjamin's(3) demonstration that

the sensitivity of sweat glands to acetylcholine is a function of local skin temperature.

The evidence suggests the possibility that the sudomotor axone reflex, should it occur physiologically, may serve to modify the response of the sweat glands to their central control. As indicated previously, however, the existence of such an axone reflex has not been demonstrated under physiological conditions.

Summary. The dose-response characteristics of nicotine axone reflex sweating are similar to those of the nicotine action on autono-

mic ganglia. The threshold for nicotine axone reflex sweating is raised with lowered environmental temperature. The effect appears to be due predominantly to alteration in local skin temperature.

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Effect of Preweanling Administration of Lactose on Subsequent Caries Susceptibility.* (23713)

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Previous work(1) showed that administration of a 20% sucrose solution 3 times a day to suckling white rats during calcification of their molars produced a highly significant increase in the subsequent caries score as compared with unsupplemented litter mates. This technic which measures only the systemic effect of supplements apart from local effects thus appeared useful in studying the systemic effect that other carbohydrates may have on subsequent caries.[†] The present study was designed to determine if lactose given under these conditions would also produce a significant change in caries.

Method. Fifty-eight animals from 7 litters were divided into 2 groups. One group of 30 animals was given 20% lactose solution orally 3 times a day, from the 3rd day following birth through the 21st day. The amount administered was approximately 1:1000 of body weight. In addition all animals received dam's milk *ad libitum*. Litter mates totaling 28 animals were used as controls and were

given nothing in addition to dam's milk throughout the suckling period. When weaned at 21 days of age, all animals were placed on a cariogenic diet of the following composition: casein 24, sucrose 65, salts USP XIV 4, corn oil 5, dehydrated whole liver 4, vitamin mix 2(2), choline 0.2 part. Animals were sacrificed at the end of 13 weeks, and the molars were examined under a 20-power dissecting microscope after grinding successive planes parallel to the occlusal surface. Individual charts which showed the relationship of enamel, dentin, and pulp of each molar were used to record the carious lesions. The lesions were scored by the method of Shaw(3). Table I shows these results.

Results. The 10% difference in caries score between experimental and control animals was not significant. This is in marked contrast to results obtained when a 20% sucrose solution was administered in equal dosage to

TABLE I. Administration of 20% Lactose and Resulting Caries Score 13 Weeks Later.

| Supplement | No. animals | Caries score (\pm S.E.) | t value |
|------------|-------------|----------------------------|---------|
| Lactose | 30 | 20.4 \pm 2.87 | .53 |
| Control | 28 | 18.2 \pm 2.88 | |

* This investigation was supported by a NIH Grant.

[†] Reported at International Assn. for Dental Research, 1957 meeting.

suckling rats. In previous work(1), 20% sucrose solution given under similar circumstances caused over a 100% increase in caries as compared with unsupplemented litter mates. A 20% glucose and fructose mixture given in a dosage of 1 to 1000 of body weight also increased the subsequent caries of white rats(4). If this increase in caries was due alone to dilution of the diets of suckling rats, the caries scores would be similar.

The results of the previous work are in agreement with findings of Sognaes(5) in which rats fed high sucrose diet during calcification of teeth had higher incidence of caries over animals not so treated. Work with monkeys also showed an adverse effect of high sucrose diet during calcification on later caries experience of teeth. Reduced caries experience of children during and after World War II also correlated with reduction in sugar imports(6). These results are in contrast with those reported in this paper in which no significant change in caries resulted from addition of lactose to diet of suckling rats during

calcification of their teeth.

Lactose is hydrolyzed more slowly than sucrose in the gastrointestinal tract. Since hydrolyzed sucrose is absorbed very rapidly, it might possibly produce a temporary mineral imbalance during the suckling period. This imbalance may be reflected in a significant increase in the caries developed later.

Summary. A 20% solution of lactose given 3 times a day for 18 days during calcification period of molars, resulted in no significant increase in caries of teeth of white rats over control litter mates.

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Excretion of Sulfated Mucopolysaccharides in Gargoylism. (Hurler's Syndrome)* (23714)

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The presence of sulfated polysaccharides has been demonstrated histologically in a number of cases of gargoylism(1) and, from their various organs, an unidentified sulfated mucopolysaccharide has been isolated(1,2). It has been established recently that the urine of 2 cases of gargoylism contained chondroitin sulfate B (ChS-B)(3,4) as well as a minor component with a positive rotation(4) designated either as heparitin sulfate(5) or heparin monosulfate(6). This paper is concerned with studies on specimens of urine from 5 patients with gargoylism and a sam-

ple of liver obtained at autopsy and preserved in a frozen state, from another patient.

Methods. The method used for isolation of sulfated mucopolysaccharides was a modification of that of Di Ferrante and Rich(7). An attempt was made to collect total urine for periods of 4 to 6 days from Cases 1-4, however, accurate collections during this entire period were not possible and the pools do not represent complete samples. The urine samples were acidified to pH 6.0 and a warm aqueous solution of 5% cetyl trimethylammonium bromide was added (1.7 ml/100 ml of urine). After standing overnight at 4° and centrifuging, the precipitate was repeatedly

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TABLE I. Mucopolysaccharide Fractions of Urine and Liver in Gargoylism.

| Source of material | EtOH fractions, yield (mg) | Analysis in % | | | | | [α] _D | Aminosugar* | |
|---|----------------------------|---------------|---------|-------|------------|-----------------|---------------------------|----------------------|-----------------------|
| | | Uronic acid | | | Hexosamine | SO ₄ | | Gal. NH ₂ | Gluc. NH ₂ |
| | | Carbazole | Orcinol | Ratio | | | | | |
| Urine of R. Do. (vol = 2.66 l) | 175 | 14.4 | 37.4 | .38 | | 15.4 | -55 | 4+ | trace |
| | 88 | 21.6 | 26.5 | .81 | | | | 2+ | 2+ |
| | 75 | 27.9 | 24.4 | 1.14 | | 11.8 | -1 | 2+ | 2+ |
| Urine of R. Br. (vol = 2.83 l) | 246† | 16.3 | 29.6 | .55 | 21.5 | 13.0 | -38 | 3+ | 2+ |
| | 71 | 31.9 | 24.5 | 1.30 | 22.8 | 15.1 | +27 | | |
| Urine of P. Gr. (vol = 1.77 l) | 243 | 12.5 | 40.6 | .38 | | 13.7 | -60 | 4+ | trace |
| | 81 | 11.2 | 21.5 | .52 | | | | 3+ | 1+ |
| Urine of J. Gr. (vol = 1.10 l) | 49 | 12.9 | 36.6 | .35 | | | | 4+ | trace |
| | 51 | 15.7 | 21.5 | .73 | | | | 3+ | 1+ |
| Urine of A. (vol = .97 l) | 77‡ | 38.8 | 19.4 | 2.0 | 24.0 | 6 | +60 | trace | 4+ |
| Liver, 73.8 g dry wt | 243 | 7.6 | 19.2 | | | | | | |
| | 442‡ | 33.7 | 17.2 | 1.96 | 24.8 | 17.4 | +61 | 0 | 4+ |
| | 974‡ | 46.6 | 17.4 | 2.68 | 28.1 | 11.0 | +69 | 0 | 4+ |
| | 335 | 38.2 | 16.3 | 2.35 | 27.1 | 13.8 | +60 | 0 | 4+ |
| ChS-B pigskin heparitin sulfate (lung) | | 16.0 | 39.1 | .41 | 24.7 | 13.4 | -63 | 4+ | 0 |
| | | 36.7 | 19.0 | 1.93 | 27.7 | 15.5 | +54 | 0 | 4+ |

* Relative amounts of glucosamine and galactosamine were determined qualitatively by paper chromatography following ninhydrin oxidation(13).

† Resistant to testicular hyaluronidase.

‡ Hydrolyzed by heparin adapted enzyme(9).

trituated with a large amount of ethanol.† Completeness of the precipitation was confirmed in some instances by dialysis of the supernatant, followed by further addition of the reagent. No further fraction was obtained. The ethanol-insoluble fraction was dissolved in 50 ml of 10% aqueous Na acetate, the pH adjusted to 9.0 with a few drops of 5 N NaOH, and any insoluble material removed by centrifugation. The residue was extracted once with 50 ml of water and the combined supernatant solutions acidified to pH 5.0. The crude polysaccharide fraction was precipitated with 1.5 volumes of ethanol. The precipitated polysaccharide was left at 4° overnight, centrifuged and washed with 80% ethanol. The fraction was extracted with a small volume of water, the extract was cleared by high speed centrifugation, Ca acetate and acetic acid were added to final concentrations of 5% and 0.5 N, respectively, and then the solution was fractionally precipitated with ethanol(8). Some of the samples were further purified by treatment with

Lloyd's Reagent and Kaolin and further fractionation(8). A slice of liver‡ was obtained frozen in dry ice. The liver was ground in a meat grinder, dehydrated and defatted by standing for several days first under acetone and then under ether. The air-dried material (73.8 g) was digested with pepsin and trypsin and fractionated as described previously (8). The methods of analysis used were previously described(8). In Table I, the data obtained from the different samples are recorded. For comparison, a typical analysis of a ChS-B fraction of pigskin(8) and one of heparitin sulfate from bovine lung§ is included.

Results. In Table I, the ChS-B fractions can be recognized by the following properties: a strongly negative rotation, a low carbazole/orcinol ratio, and resistance to testicular hyaluronidase, while the heparitin fractions show a positive rotation, contain glucosamine

‡ We are greatly indebted to Dr. G. A. Jarvis for this autopsy specimen and for urine from Case No. 5.

§ The heparitin sulfate(5) was a side product in production of heparin. It will be described later. We thank the Upjohn Co. for kindly supplying a sample.

† A large volume of ethanol, approximately 200 ml, is important since the complex is soluble in low alcohol concentration.

instead of galactosamine, have a high carbazole/orcinol ratio, and are resistant to testicular hyaluronidase. In addition, they were digested by an enzyme obtained from a micro-organism adapted to heparin(9). The urine of Case #1 yielded 175 mg or 66 mg per 1000 ml of a fraction with the typical solubility and properties of ChS-B. On increasing the alcohol concentration, 2 additional fractions were obtained representing a polysaccharide mixture of approximately the same quantity, half of which was probably heparitin sulfate. The remainder has not been identified. From the urine in Case #2, 246 mg or 87 mg per 1000 ml of an impure fraction were obtained which, on the basis of analysis and resistance to testicular hyaluronidase, consisted mainly of ChS-B. The second fraction, obtained by increasing the ethanol concentration (71 mg or 25 mg per 1000 ml), was mainly heparitin sulfate. Cases #3 and #4 were siblings. The girl (Case #4), who had as yet only minimal symptoms of the disease, excreted 49 mg or 45 mg per 1000 ml of ChS-B and 51 mg of a mixture containing apparently only a small quantity of heparitin sulfate, while the remainder was a chondroitin sulfate of undetermined type. The older brother, (Case #3) who had a more advanced form of the disease, excreted 243 mg or 137 mg per 1000 ml of ChS-B and, in addition, 81 mg of a fraction similar in composition to that of his sister.

From the urine of Case #5 no ChS-B was obtained, and only 77 mg, or 79 mg per 1000 ml of a heparitin sulfate fraction. This fraction, however, showed an unexplained low sulfate value. From the liver of Case #6, a total of 1.75 g or 2.37% of the dry weight of the defatted tissue was isolated and identified as heparitin sulfate. In addition, 243 mg of a crude polysaccharide was obtained which, on refractionation, did not contain a significant amount of ChS-B.

In addition, 24 hour specimens of urine from the father (1790 ml) and mother (1260 ml) of Cases #3 and #4 were examined. These urines yielded 25 mg and 40 mg, respectively, of very impure fractions containing, by analysis, only 6 to 10 mg of unidentified sulfated polysaccharides. As additional controls, 3- and 5-day collections of children

of 10 and 1½ years of age, admitted to the hospital for minor surgical procedures, were collected. In identical procedures of precipitation and fractionation, the urines (1.8 l and 3.9 l, respectively) yielded 20 and 30 mg, respectively, of sulfated polysaccharide with a normal ratio of carbazole to orcinol. No precipitate was obtained at 20% EtOH. We conclude from these data that these urines contained no demonstrable quantities of ChS-B. From normal urine, chondroitin sulfate fractions (7-8 mg/day) hydrolysable by testicular hyaluronidase were isolated(7). ChS-B or heparitin sulfate has not been found in normal urines. Four of the 5 patients with gargoylism excreted large amounts of sulfated mucopolysaccharides which consisted mainly of ChS-B. In addition, 4 cases excreted at least one other mucopolysaccharide fraction, apparently identical with heparitin sulfate. The latter varied in concentration from approximately 10% to 70% of the major fraction. One patient excreted only heparitin sulfate. From the liver of one autopsied case, large quantities of heparitin sulfate were isolated. A polysaccharide of apparently similar nature was isolated from the liver of a case of gargoylism by Stacey and Baker(10). In none of the patients' urines were significant quantities of ChS-A or C detected, which, at least in young individuals, are the main sulfated mucopolysaccharides of connective tissue. Table II contains the significant clinical data for each of the patients and the type of mucopolysaccharide isolated.

Discussion. ChS-B occurs normally in all connective tissue with the exception of cartilage, bone and cornea, while heparitin sulfate has been isolated only from lung, aorta, and in large quantities from amyloid liver. Dorfman and Lorincz also isolated ChS-B and a heparitin-like fraction from the urine of a female patient with gargoylism. Hence, the excretion and storage of these mucopolysaccharides appear to be a distinctive feature of this disorder.

The evidence for a genetic origin of gargoylism has been comprehensively reviewed by McKusick(11) who suggests that there are at least 2 distinct genotypes—a sex-linked recessive and an autosomal recessive form.

TABLE II. Clinical Findings of the Cases of Gargoylism Studied.

| Pa- tient | Age (yr) | Sex | Race | "Gargoyle habitus" | Skeletal lesions | Mental retarda- tion | Corneal opacities | Hepato- spleno- megaly | Cardiac abnor- malities | Gibbus | Muco- polysaccharides |
|--------------|-------------------------------|-----|------|-----------------------|---------------------|----------------------------|----------------------|------------------------------|-------------------------------|--------|---|
| 1.† | 6 | ♂ | N | ++++ no dwarfism | ++ | ++ | 0 | +++ | ++ | 0 | Urine, Ch-SO ₄ B, Hep-SO ₄ |
| 2.† | 12 | ♂ | N | ++++ | ++ | + | ± | +++ | +++ | 0 | <i>Idem</i> |
| 3.* | 8 | ♂ | W | ++++ | +++ | ++ | +++ | +++ | ± | + | " |
| 4.* | 1 ¹ / ₂ | ♀ | W | + | + | + | ++ | + | 0 | 0 | " |
| 5. | 7 | ♂ | W | ++ | ++ | ++ | 0 | yes | ? | ? | Urine, Hep-SO ₄ only |
| 6.‡ | 10 | ♂ | W | ++++ | +++ | +++ | 0 | " | " | " | Liver, Hep-SO ₄ only |

Severity of lesions: 0 - + + + +. ±, cardiac abnormalities: organic murmur and cardiac enlarge-
ment.

* Sibling pair; 2 normal male siblings.

† History of a similarly affected maternal uncle and
normal female sibling(s).

‡ Parents' first cousins; affected male siblings.

The pedigrees of Cases #1 and #2, and of Cases #3 and #4, respectively (Table II), are compatible with these two different modes of inheritance. An analysis of the chemical findings in these 4 patients, according to the postulated genotype, did not show an obvious difference in the pattern of urinary mucopolysaccharides.

It is difficult to explain gargoylism in terms of a single metabolic defect since two mucopolysaccharides of different composition and structure appear to be involved. Further, the absence, in these cases, of significant quantities of other mucopolysaccharides excreted in the urine of normal individuals, such as ChS-A and C, and hyaluronic acid, appears to exclude a general aberration in the metabolism of mucopolysaccharides, hexuronic acids, hexosamines or sulfate.

In view of the large quantities of mucopolysaccharides excreted in the urine, it seems probable that in gargoylism these substances are produced in excess. Other possibilities include an inability to bind the polysaccharides at their normal sites or a defect in their metabolic breakdown.

It has been pointed out previously (8) that the mucopolysaccharides produced by connective tissue in different sites fall into distinct patterns. Furthermore, tumors of mesodermal origin contain only one mucopolysaccharide and not mixtures of various polymers (8). Therefore, it seems possible that connective tissue cells each produce only one specific type of product, *e.g.*, cells producing

hyaluronic acid cannot produce any of the chondroitin sulfates. A tentative explanation of what appears to be an overproduction of certain normal polysaccharides in gargoylism might be sought in a genetically determined error of differentiation of the fibroblasts. The excessive production of polysaccharides results in the accumulation of these substances in connective tissue cells as well as the cells of many organs and the excretion of large quantities in the urine.|| This hypothesis is consonant with the pathologic observations of Lindsay (12), *et al.* and others (see 11) which suggest that gargoylism is both a generalized disorder of the connective tissue and a storage disease. The precise nature of these two aspects has not been defined.

Summary. 1. From the urine of 4 out of 5 patients with gargoylism, a mixture of mucopolysaccharides was isolated which could be identified as ChS-B and heparitin sulfate—with the former predominating. The urine of one patient, and the liver of another, yielded only heparitin sulfate. 2. The type of polysaccharide excreted by these patients had no obvious correlation with the severity of the disease or the postulated mode of inheritance suggested by the history. 3. It is suggested that gargoylism represents an overproduction of certain mucopolysaccharides due to a genetically determined error of differentiation

|| Storage of lipid, especially in brain and, in part, in liver has been regarded by Uzman (2) and by Brante (1) as a secondary effect which results from the derangement of normal cellular function.

of fibroblasts.

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Failure to Sensitize to Autologous Skin.* (23715)

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(Introduced by M. B. Sulzberger) (With technical assistance of Gerald Kaplan)

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The possibility that human beings could develop allergic sensitivity to their own skin, either alone or in combination with foreign materials, has been considered for many years. In clinical dermatology this concept is so ingrained that the diagnoses of autosensitization dermatitis and autoeczematization are in common use in some dermatological departments. Detailed descriptions of the phenomena which denote "autosensitization" have been given (Whitfield(1), Haxthausen (2)) and studies by means of skin and serologic tests using extracts of skin have been carried out (Templeton(3), Cormia(4)). There is, however, a scarcity of basic immunologic studies in this highly important segment of dermatologic investigation. Hecht, Sulzberger and Weil(5) produced precipitating antibodies in rabbits against homologous skin by injecting them with skin adsorbed on aluminum cream, and with staphylococcus toxin. They found that rabbits with these antibodies in their blood developed skin lesions when their skin was subjected to certain non-specific traumas, while rabbits not

having such circulating antibodies did not react in such a manner. Voisin and Maurer (6) injected 6 rabbits with homologous skin and adjuvants. When subsequently they grafted the injected rabbits with skin from the donor animals, they observed the development of peculiar cutaneous lesions. Such lesions did not develop in animals which had received the skin-adjuvant mixture, but no skin grafts. Furthermore, 4 of the 6 test animals subsequently rejected autografts, an event which Voisin and Maurer attributed to autosensitization to skin.

In the present studies in guinea pigs we used technics similar to those which have been reported to have been successful in engendering experimental sensitization to homologous or autologous organs in experimental animals, using a variety of tissues: kidney(7), lens(8), nervous tissue(9-12), tumor(13) and testes (14).

Method. Emulsions of autologous skin were prepared as follows: guinea pigs, after having been clipped and shaved on the flank using a germicidal soap lather, were placed under general anesthesia with sodium pentothal. After sponging the skin with 0.1%

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cetyl trimethylammonium bromide in 70% ethyl alcohol, a full thickness piece of skin was removed from the prepared area of each animal by raising the skin with a forceps and slicing across with a razor blade. The skin sample obtained was oval in shape and averaged about 20 mm long, 15 mm wide, 2 to 3 mm thick at the center and 300 to 400 mg in weight. The skin was weighed to the nearest 0.1 g, minced with scissors and added to 4 volumes of sterile saline or staphylococcus toxin diluted 1:5 with saline. This crude skin suspension was fragmented in a high speed homogenizer† for 10 minutes while being cooled in an ice-bath. The resulting 'skin broth' except for a few remaining larger fragments of skin was taken up through a 20 or 21 gauge needle and emulsified in a syringe with 2 volumes of a mixture of Bayol F and Arlacel A containing 1 mg of killed, dried tubercle bacilli‡ per ml. The ratio of Bayol F to Arlacel A was either 8½:1½ or 2:1. Thus each ml of skin-adjuvant emulsion contained approximately 67 mg of skin. Skin-adjuvant emulsions which were used for more than 1 injection were kept in a freezer at a temperature of approximately -12°C, but none were kept longer than 1 week. Test animals received injections of their autologous skin emulsion with adjuvants, while control animals were given an emulsion of saline or staphylococcus toxin diluted 1-5 with saline, with adjuvants. Injections of skin-adjuvant emulsions were made intraperitoneally (0.1-0.3 ml), intracutaneously into multiple sites on the back (0.3-0.6 ml) or intramuscularly into the limbs (2 ml). A total of 89 guinea pigs were injected with autologous skin emulsion and 38 with control emulsion. Animals were autografted 28 to 64 days after the first injection of skin or control emulsion, following closely the pinch graft technic of Billingham and Medawar(15). All autografts were taken from sites which previously had been tattooed with India ink, in order to permit easier identification of accepted grafts. Grafts

TABLE I. Results of Autografts in Guinea Pigs Injected with Autologous Skin in Emulsion with Adjuvants.

| Route of inj. | Wt of skin inj., mg | No. of successful autografts/autografts done | | | |
|---------------|---------------------|--|------|-----------------|-----|
| | | Test animals | | Control animals | |
| Intraeut. | 20-40 | 32/34 | 94% | 19/21 | 90% |
| Intramuse. | 134 | 28/30 | 93% | 16/17 | 94% |
| Intraper. | 7-27 | 25/25 | 100% | Not done | |

were taken from the right posterior flank and placed in a prepared site on the center of the side of the thorax. The graft, after it had been placed in its bed, was covered with sterile, petrolatum-impregnated gauze, followed by a dry gauze pad. Finally a plaster cast was applied surrounding the entire thorax to prevent the graft from slipping from the bed. The grafted sites were inspected at 7, 10, 14, 21, and 28 days, or until the graft had permanently healed in or had been rejected. In some instances, after the autologous skin graft experiment had been concluded, the skin of both test and control animals was tested for its response to physical trauma, as follows: one flank of the animal was clipped and traumatized by 1) superficial scraping of a skin area approximately 2 cm² using 10 strokes of a sharp scalpel blade, and 2) by burning another skin site using the Henriques-Moritz burn apparatus at 100°C for 60 seconds. Reactions to these forms of physical trauma were observed after 1, 2, and 7 days.

Results. The results of the autograft experiments are summarized in Table I. This indicates that there was no significant difference in the percentages of skin autografts accepted and rejected between the animals pretreated with an emulsion of their own skin and control animals. It may be assumed that the small percentage of failures to accept autografts in both groups was due to technical factors rather than specific immunologic alterations. The tests with physical trauma did not produce any unusual reactions, no significant differences being noted in the response between the test and control animals. Thus neither the autograft experiments nor the irritation tests produced any evidence indicating that sensitization to autologous skin had taken place after the intracutaneous, in-

† "Vir-Tis 45" made by the Vir-tis Co., Yonkers, N. Y.

‡ The tubercle bacilli were kindly supplied by Dr. Jules Freund.

tramuscular or intraperitoneal injections of autologous skin and adjuvants.

Summary. 1) Eighty-nine guinea pigs were injected intracutaneously, intramuscularly or intraperitoneally with autologous skin in emulsion with adjuvants. Thirty-eight guinea pigs were injected intracutaneously or intramuscularly with control emulsion, not containing skin. Subsequently an autograft was done on each animal. No significant differences were noted in acceptance of autografts between animals injected with skin emulsion and control emulsion. 2) Irritancy tests using scraping and burning of the skin did not produce different results in the test animals and the control animals. 3) Under the conditions of these tests no autosensitization to skin could be demonstrated in guinea pigs.

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Chromatographic Differences Between Radioiodinated Albumin Preparations and Normal Human Serum Albumin. (23716)

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Radioiodinated human serum albumin has been extensively used to investigate albumin metabolism in the normal and in diseased states(1-3). Albumin preparations used in these studies have been indistinguishable from normal human serum albumin in many respects but not in all(1,2,4,5). The recent introduction of anion-exchange adsorbents(6) which have proved very useful in the chromatographic separation and characterization of serum proteins(7,8), made possible further evaluation of radioiodinated human serum albumin preparations. In the present study, utilizing the anion-exchange adsorbent, diethylaminoethyl-cellulose(6), the chromatographic distribution of radioiodinated human serum albumin was investigated and found to differ markedly from the distribution of na-

tive serum albumin.

Materials and methods. The radioiodine-labeled human serum albumin preparation used in these studies has been commercially prepared by the following procedures. Albumin was separated at the Cutter Laboratories from pooled plasma by a modification of the cold ethanol technic of Cohn(9). Following addition of stabilizers (acetyltryptophan and sodium caprylate) the albumin was heated to 60°C for 10 hours to minimize the possibility of transmission of homologous serum hepatitis(10). Iodination of the albumin was performed at the Abbott Laboratories(11) and included exposure of the albumin to potassium iodide and sodium hypochlorite solutions and passage over an ion-exchange column. This preparation, referred

to as RISA-T₃, contained 1 atom of iodide for every 5.5 molecules of albumin. Half a ml of this iodinated albumin solution, containing 25 μ c and 25 mg of albumin, was added to 25 ml of fresh normal human serum containing 950 mg of albumin. Anion-exchange (DEAE-) cellulose chromatography was carried out as described in detail elsewhere(8). The solution containing I¹³¹-labeled albumin and serum was dialyzed for 21 hours at 4°C with several changes of the 0.01 M pH 8 sodium phosphate buffer, and 20 ml of the dialyzed preparation was added to a column containing 16 g of DEAE-cellulose adsorbent.* Gradient elution with a flow rate of 20-25 ml/hr was employed with one liter of 0.01 M pH 8 sodium phosphate buffer in the mixing chamber and 500 ml of 0.30 M monosodium phosphate in the reservoir. Effluent fractions of 8 ml were collected and examined for protein content by measurement of the optical density at 280 m μ in a Beckman DU spectrophotometer. Electrophoretic identification(8) of the chromatogram fractions was performed after ultrafiltration. Total protein determinations were made by standard biuret procedure(8). Radioactivity of 2 ml aliquots of the chromatographic fractions was measured in a thallium-activated sodium iodide well scintillation counter. Localization of the radioactivity in the whole serum and chromatographic fractions was ascertained after paper electrophoresis by means of an end window Geiger-Muller counter, employing a continuously recording counting-rate meter, and the distribution of radioactivity was compared with protein distribution, as determined by bromphenol blue staining. Radioactivity was determined on duplicate unstained and stained strips without detection of significant difference.

Results. The chromatographic distribution of the normal serum proteins, of serum albumin and of radioiodinated albumin are illustrated in Fig. 1. The pattern of the serum chromatogram is typical of that obtained with normal serum when this technic is employed,

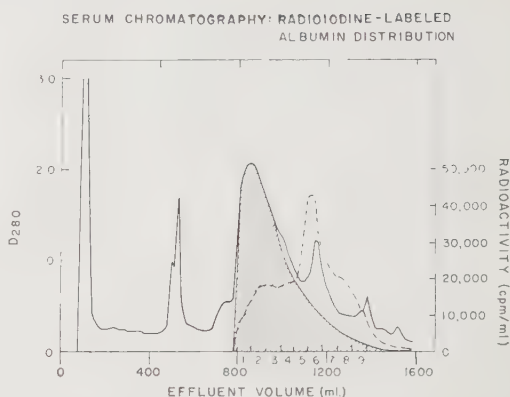


FIG. 1. Chromatogram of normal human serum and I¹³¹ albumin. Protein distribution is indicated by solid line, albumin distribution by shaded area, and radioactivity (I¹³¹ albumin) by broken line. The radioactive albumin represents only a very small fraction (less than 1%) of the total amount of albumin in the serum. The serum proteins distribution pattern is normal(8). The near coincidence of the major peak of radioactivity elution with a peak of serum protein elution is probably fortuitous for this serum protein peak is normally present and contains largely alpha-1, alpha-2 and beta globulins.

and the distribution of native serum albumin also agrees with previous experience(8). However, the difference between serum albumin and radioiodinated albumin distribution is readily apparent.

The effluent region containing almost all of the radioactivity (800 to 1400 ml) was subdivided into 9 equal parts. Aliquots from the effluent fractions in each part were pooled, concentrated by ultrafiltration and examined by electrophoresis. There was no demonstrable radioactivity in the ultrafiltrate. After electrophoresis of these pools the distribution of radioactivity was found to coincide with the location of albumin in every instance. The quantity of albumin and radioactivity in each of these regions are presented in Table I. Combined fractions 1-4 were composed almost entirely of albumin yet were of low specific activity. Although the greatest radioactivity (I¹³¹ albumin) was found in combined fractions 5 and 6, there was a further relative increase in radioactivity in pools 7-9 as the total albumin content decreased further in the final portions of the chromatogram. Fig. 2 illustrates the electrophoretic protein and radioactivity distribution in the regions where albumin elution was greatest (865-928 ml)

* We are indebted to Dr. E. A. Peterson and H. A. Sober of the Laboratory of Biochemistry, Nat. Cancer Inst., for the DEAE-cellulose (1.3% nitrogen) utilized for this study.

TABLE I. Distribution of Albumin and Radioactivity in Combined Fractions of Chromatogram Effluent.

| † | Combined fraction Effluent vol (ml) | Albumin | | Radioactivity | |
|---|--|----------------------|----------------------|---------------|-------------------------------------|
| | | % of protein present | Amt of albumin* (mg) | Total cpm | cpm/mg albumin ($\times 10^3$) |
| 1 | 801- 864 | 98 | 173 | 469 | 2.7 |
| 2 | 865- 928 | 99 | 191 | 936 | 4.91 |
| 3 | 929- 992 | 100 | 156 | 951 | 6.07 |
| 4 | 993-1056 | 98 | 103 | 1003 | 9.73 |
| 5 | 1057-1120 | 90 | 63 | 1543 | 24.49 |
| 6 | 1121-1184 | 53 | 41 | 1775 | 43.40 |
| 7 | 1185-1248 | 70 | 29 | 1188 | 40.95 |
| 8 | 1249-1312 | 74 | 21 | 964 | 45.90 |
| 9 | 1313-1376 | 21 | 7 | 499 | 71.31 |

* Obtained by determining total protein content (biuret) and albumin content by electrophoresis.

† Chromatogram region indicated on Fig. 1.

and where the radioactivity elution was greatest (1121-1184 ml). The total recovery of I^{131} albumin from the column was 91% of the amount applied.

Discussion. The difference observed in the present studies, between the chromatographic distribution of iodinated albumin preparations and native human serum albumin, was marked and striking. Two other iodinated albumin preparations have been similarly chromatographed and the distribution of iodinated albumin in each instance was found to resemble that reported here. Whether this change is attributable to the addition of radioiodine or to some step in the separation and sterilization of albumin remains to be determined.

Oxidizing agents such as hydrogen peroxide, used for converting iodide to iodine in I^{131} albumin manufacture, have been shown to change the biologic properties of rabbit albumin(12). It cannot be determined from the present study whether the 5-fold excess of hypochlorite is responsible for the changes observed.

Certain immunochemical and biochemical properties of human or mouse serum albumin may be altered following iodination to a level averaging 15 or more iodine atoms per albumin molecule.† However, in the RISA-T3 preparation used in this study only one iodine atom was present for each 5.5 molecules of albumin making it unlikely that excessive io-

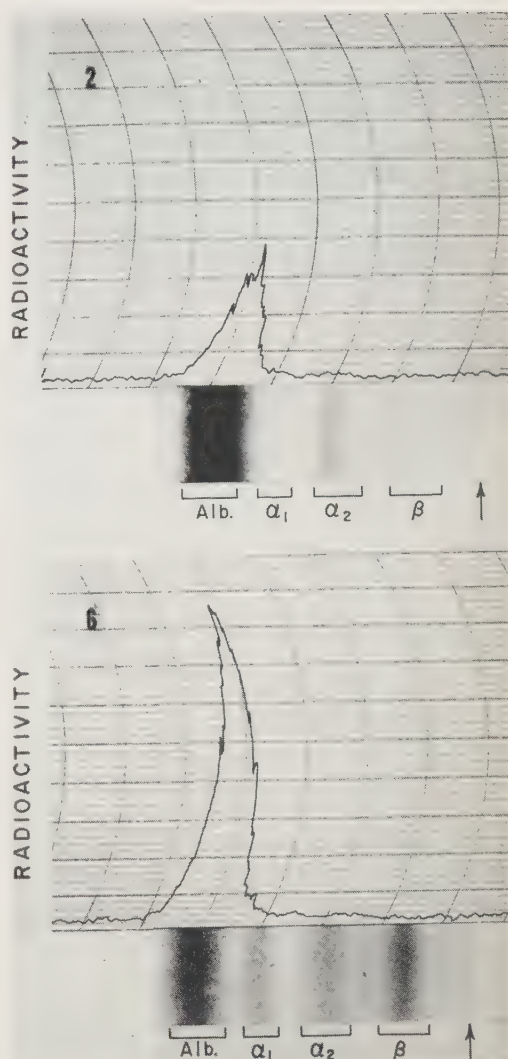


FIG. 2. Electrophoretic protein and radioactivity distribution are compared in chromatogram regions of greatest albumin content (Region 2) and of greatest radioactivity content (Region 6).

dination is the reason for the differences between native and iodinated albumin shown in Fig. 1. Although on the average there is less than one iodine atom per albumin molecule it is possible that heavy iodination of some albumin molecules may have altered their chromatographic properties. It is also possible that iodination occurred primarily on albumin molecules with distinctive chemical and chromatographic properties, either normally present or produced during albumin separation and sterilization. Iodination in-

† Steinfeld, J. L., unpublished observations.

creases the dissociation of the phenolic hydroxyl groups(13). The resultant increased acidity of the iodinated molecules may be responsible for the separation from the bulk of non-iodinated molecules on the DEAE-cellulose column.

Radiation may alter albumin. A total dose of 50,000 roentgen equivalent physical (rep) to albumin in a solution of 0.2 mg albumin/ml can alter its biologic properties(14). However, increasing the albumin concentration to 5 mg/ml partially protects against the damaging effects of irradiation. The RISA-T3 preparation used here was exposed to a relatively low radiation dose, receiving approximately 10,000 rep in a solution containing 50 mg of albumin per ml. Also, the biologic characteristics of this I^{131} albumin preparation compare favorably with those previously reported(2). Following intravenous administration to man of RISA-T3 preparations, a constant rate of iodinated albumin degradation during 30-day periods of observation has been repeatedly observed.[†] This would suggest that the iodinated albumin preparation that was chromatographed had not been severely damaged by radiation, for radiation damaged albumin preparations contained variable amounts of rapidly degraded iodinated albumins.

Studies to determine which of the many possibilities discussed above were responsible for altering the iodinated albumin so that its chromatographic behavior differed from that of native serum albumin are being undertaken. Further, the significance of these chro-

matographic differences in terms of biologic properties remains to be elicited.

Summary. The chromatographic behavior of radioiodinated albumin preparations was found to differ markedly from that of native serum albumin on anion-exchange (DEAE-) cellulose adsorbent columns. The significance of this observation is discussed.

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Effect of p-Dimethylaminoazobenzene and β -Naphthylamine on Rat Liver Sulfhydryl Concentration.* (23717)

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Various experimental procedures and numerous agents will influence the total non-protein sulfhydryl (TNPSH) content of rat and mouse livers(1-8). Scalding, ligation

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trauma, exposure to cold or shock, restraint and subcutaneous injections of adrenalin, have been shown to cause a marked drop in the total non-protein sulfhydryl concentration. Over the past 2 years(9,10), research conducted in this department has revealed

that nonnarcotic analgesics such as acetylsalicylic acid, salicylamide and acetophenetidin inhibited the decrease of the total non-protein sulfhydryl content in the liver of the rat subjected to stress. Aminopyrine and sodium salicylate failed to inhibit the decrease of this sulfhydryl content of the stressed rat liver. Early reports(11-15) suggested the interaction of carcinogens with SH-groups of cell proteins and particularly of enzyme proteins, and recent investigations(16-19) pointed out that carcinogenic agents must in some way interfere with cell constituents in order to exert their cytochemical effects. Therefore, a study was made of the influence of carcinogenic agents on the total non-protein sulfhydryl concentration of the rat liver.

The purpose of these studies was 3-fold: (1) To compare the effects of stock and low protein (8%)-low riboflavin diets on the total non-protein sulfhydryl (TNPSH) content of the rat liver; (2) to determine the changes induced in the total non-protein sulfhydryl level by the addition of p-Dimethylaminoazobenzene (DAB), commonly known as "Butter Yellow" and β -Naphthylamine (BNA), designated as 2-Naphthylamine, to these diets; (3) to ascertain whether or not there is any correlation between the liver non-protein sulfhydryl concentration and phases of carcinogenesis under these experimental conditions.

Materials and methods. The method for determining the TNPSH was basically that developed by Kolthoff and Harris(20), as modified by Benesch and Benesch(21,22) and applied by Takesue and Miya(10). The animal is sacrificed by cervical dislocation. The liver is removed and immediately frozen in dry ice. A portion (440-425 mg) is cut and weighed. This tissue is treated with 10 parts of 2.5% sulfosalicylic acid in an all glass tissue grinder and ground quickly. After centrifugation and filtration through shark-skin filter paper, a 1 ml aliquot is placed in the titration mixture consisting of not less than 85% ethanol, with the addition of sufficient ammonium hydroxide and ammonium nitrate to make the concentrations 0.25 M and 0.05 M respectively. The titration mixture is made 3×10^{-5} N in ethylenediamine

TABLE I. Effects of Fasting and Acute Doses of Carcinogenic Agents on the TNPSH Content of the Rat Liver.

| No. of animals | Treatment | Avg, mg %* | \pm S.E. |
|----------------|---|------------|------------|
| 6 | None | 302.8 | 5.2 |
| 10 | Fasting (F), 20-24 hr; water <i>ad lib</i> † | 282.7 | 3.0 |
| 4 | F; DAB, 10 mg/kg | 300.0 | 5.3 |
| 4 | 20 | 274.0 | 9.7 |
| 4 | 50 | 259.0 | 12.6 |
| 4 | F; BNA, 5 | 272.0 | 5.9 |
| 4 | 10 | 201.2 | 6.2 |
| 5 | 20 | 208.5 | 3.0 |

* Avg of duplicate titrations.

† Used as controls for subsequent work.

tetraacetate tetrasodium salt. The mixture is titrated at the rotating platinum electrode with 0.0005N silver nitrate solution. The theoretical end point of the titration is obtained graphically by plotting current readings (microamperes) against volume of silver nitrate solution added and noting the intersection of the two straight lines. The results are presented as mg% of TNPSH calculated on the wet weight of the liver. The TNPSH content of the liver of normal fed and fasted (20-24 hours) female Sprague-Dawley rats was determined amperometrically. The values found agree quite well with those reported by Takesue and Miya(10).

Results. Acute experiments. Groups of 4-5 female Sprague-Dawley rats, fasted for 20-24 hours with water *ad libitum* prior to the experiment, received 10 mg, 20 mg, 50 mg, per kg of DAB and 5 mg, 10 mg, 20 mg, per kg of BNA respectively. The carcinogenic agents were suspended in a 0.2% solution of sodium alginate in water and administered orally by means of a stomach tube. All concentrations of the carcinogens were suspended in this agent in such a way that the volume of solution administered to the animals remained constant. Four to 5 hours after the gavage, the rats were sacrificed and the sulfhydryl determination made.

The results, Table I, indicate that fasting alone (20-24 hours) had very little effect on the TNPSH content of the rat liver. In acute experiments, DAB, administered up to 50 mg/kg, failed to produce a marked decrease

in the sulfhydryl level, whereas BNA, given at a dose of either 10 mg or 20 mg/kg, induced a significant and similar drop in the TNPSH concentration. A dose of 5 mg/kg of BNA was without effect.

Chronic experiments. The main objective of this problem was to determine the changes induced in the total non-protein sulfhydryl (TNPSH) level by addition of p-Dimethylaminoazobenzene (DAB) and β -Naphthylamine (BNA) to experimental diets. Reports have emphasized the importance of the diet and its riboflavin content during the process of liver cancer (23,24). Consequently, diets having a well-defined composition from a nutritional standpoint, have been used throughout the entire experiment for the purpose of studying primarily the influence of DAB and BNA on the sulfhydryl level of the rat liver.

Female Sprague-Dawley rats, weighing approximately 140 g at the onset of the experiment, were maintained on 2 different diets: a stock Purina Laboratory Chow powder and a low protein (8%) diet[†] with a low riboflavin content (2 mg/kg diet). In each instance, the carcinogens were incorporated thoroughly in the food, 600 mg of DAB or 100 mg of BNA per 1 kg of diet. To secure uniform distribution of the carcinogens in the diets, mechanical mixing of at least 1 hour was provided by an electrical ribbon mixer. These diets were fed to the rats daily and no measurement of the individual food intake was made. In order to prevent accumulation of old food, the food cups were refilled each day. The average food consumption was good and the weight loss recorded was not due to refusal of rats to eat the experimental diets. For each group of rats fed a diet containing DAB or BNA, control animals were fed the same diets without the carcinogens. The feeding period of the deficient diet was 16 weeks and the normal diet 24 weeks. All animals were weighed weekly and their weight recorded. At the beginning of the experiments the average weight of the animals in all groups was about 140 g; at 16 weeks average weights in the various groups were as follows: Normal diet, 247; low protein diet, 214; low protein

and DAB, 167; low protein and BNA, 214, normal diet and DAB, 229; normal diet and BNA, 257. The animals on the low protein diet plus DAB lost weight at the 6th week and gained the least during the experimental period. To determine under such well defined nutritional conditions the effects of the carcinogens on the sulfhydryl content, 5 animals of each control and experimental group were selected at random every 4 weeks and the TNPSH concentration determined as previously described. Food was withheld from the animals 20-24 hours with water *ad libitum* prior to the experiment. At the time of sacrifice, portions of the liver, adrenal, spleen, pancreas, kidney and bladder of each rat were fixed for histopathologic examination.

Table II gives a summary of the liver sulfhydryl level after prolonged treatment. The data, from the chronic experiments, revealed that the TNPSH level of the stock diet fed rats remained relatively constant throughout the experiment. The low protein diet fed rats showed a decrease after 12 weeks but the liver TNPSH values returned to near normal in 16 weeks. There is also indication that the addition of 600 mg of DAB or 100 mg of BNA per 1 kg of diet decreased the TNPSH concentration of the rat liver. The incorporation of BNA to a non-protective diet caused a maximum drop after 4 weeks. Under the same experimental conditions, the effect of DAB was quite marked after 8 weeks with very little variation at the end of 16 weeks.

These carcinogens, when added to the stock diet, likewise decreased the liver sulfhydryl level. In this instance, the results indicate that both DAB and BNA induced their maximum decrease after 12 weeks, with a gradual return to normal values at the 20th week.

Tissue Pathology Studies.[‡] Examination of the tissues, at the time of sacrifice, showed a few cases of enlarged spleens and kidneys and no gross changes in the pancreas, bladders, and adrenals of the experimental animals. The livers of the deficient diet-DAB fed rats exhibited more severe gross pathologic

[‡] The authors are indebted to Dr. A. L. Delez, Veterinary Science Dept., for interpretation of the histopathologic sections.

[†] Nutritional Biochemicals Corp., Cleveland, O.

TABLE II. Effects of Carcinogenic Agents on TNPSH Content of Rat Liver.*

| Diet | Weeks | | | | | |
|----------------------|-------|-------|-------|-------|-------|-------|
| | 4 | 8 | 12 | 16 | 20 | 24 |
| Normal, control | 281.5 | 285.0 | 285.0 | 281.0 | 295.0 | 290.0 |
| | 2.9 | 6.0 | 3.1 | 4.0 | 4.0 | 7.6 |
| Low protein, control | 281.0 | 271.5 | 221.0 | 275.0 | | |
| | 3.2 | 10.3 | 6.7 | 8.1 | | |
| Low protein & DAB† | 279.5 | 198.0 | 181.0 | 193.0 | | |
| | 9.4 | 12.4 | 5.7 | 11.5 | | |
| Low protein & BNA‡ | 181.5 | 222.5 | 200.0 | 230.0 | | |
| | 14.9 | 6.0 | 2.0 | 6.1 | | |
| Normal & DAB† | 307.0 | 293.0 | 218.5 | 265.0 | 314.0 | 263.0 |
| | 5.3 | 5.3 | 6.3 | 17.8 | 2.4 | 5.5 |
| Normal & BNA‡ | 282.0 | 272.0 | 225.0 | 257.0 | 304.0 | 282.0 |
| | 13.3 | 14.9 | 8.0 | 8.0 | 6.0 | 8.4 |

* Avg mg % (\pm S.E.).
added to 1 kg of diet.

† 600 mg of DAB added to 1 kg of diet.

‡ 100 mg of BNA

changes than those of the stock diet-DAB fed rats. The pancreas, spleens, adrenals, kidneys and bladders had no evidence of significant histopathologic changes.

Significant tissue abnormalities occurred in the livers of rats under DAB treatment. A severe degeneration of the parenchymal cells was observed in the low protein-diet fed rats. This lesion is associated with the appearance of abnormal parenchymal cells which have large hyperchromatic nuclei. These features may represent the early stages of neoplastic formation in the organ.

Discussion. The exact mechanism by which these carcinogens exert their effects on the TNPSH content of the rat liver, when added to a diet, is unknown. The results suggest that the low protein diet creates a type of stress which manifests itself in a decrease in the liver TNPSH content and that the carcinogenic agents will augment the severity and the acuteness of the stress condition. It is also obvious that DAB and BNA act independently of the stress state induced by the non-protective diet, causing a sulfhydryl depletion even with the stock diet. The sulfhydryl compounds, as Barron(25) pointed out, are indispensable requirements for processes of growth, enzyme activity and metabolism. It is reasonable to assume that a detoxication of the carcinogens, which takes place in the body, requires and binds sulfhydryl groups, in this manner depriving the organism of a vital factor of normal growth.

Whether or not the sulfhydryl derivatives, which we assume to be formed in the body, may be intermediates in either the detoxification mechanism, the process of carcinogenesis, or both remains to be elucidated. The fact that the TNPSH level of the low protein diet control group at 12 weeks was significantly low indicates that the diet might have played an important if not a primary role in TNPSH depletion. Indeed, it has been shown that liver tumor growth is facilitated by such diets.

Summary. An investigation was made of the effects of 2 carcinogenic agents on the total non-protein sulfhydryl content of the rat liver. It was found that fasting decreased the sulfhydryl content only slightly. Acute doses of β -Naphthylamine produced a marked lowering of the total non-protein sulfhydryl concentration, while p-Dimethylaminoazobenzene failed to elicit this effect. It was possible to demonstrate that the total non-protein sulfhydryl level of the stock diet fed rats remained relatively constant throughout the experiment and that low protein-low riboflavin diet created a type of stress which manifested itself in a decrease in the total non-protein sulfhydryl compounds of the rat liver. This study indicates that addition of 600 mg of p-Dimethylaminoazobenzene or 100 mg of β -Naphthylamine/kg of diet induced a decrease in the total non-protein sulfhydryl content of the liver. The sulfhydryl depletion, which takes place under such experimental conditions, may be involved in either the detoxica-

tion mechanism, the process of carcinogenesis, or both. However, it is to be emphasized that the data do not permit any conclusion with respect to the relationship between carcinogenesis and liver sulphydryl concentration.

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Acute Choline Deficiency in Albino Rats: Vascular Contractility, Vascular Fragility, and Blood Pressure.* (23718)

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The rapidly developing and spectacular effects of choline deficiency in the weanling albino rat have been described (1-6). The most conspicuous feature of the deficiency syndrome is hemorrhagic degeneration of the kidneys, although hemorrhages are also observed in many other organs including eyes, heart, adrenals, liver and brain. The present study is concerned with an attempt to quantitate changes in vascular contractility and fragility during the syndrome, by technics to be described, and includes observations of blood pressure made during the course of the investigation.

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Methods. Composition of deficient diet was: casein 18%; lard 30%; cornstarch 30%; sugar 12%; agar 2%; salt mixture 4%; cod liver oil 4%; wheat germ oil 1%; salad oil 1%; and thiamine-8 mg, riboflavin-8 mg, pyridoxine-8 mg, calcium pantothenate-15 mg, nicotinic acid-15 mg, nicotinamide-15 mg/kilo of diet. A preliminary trial demonstrated that this diet produced typical findings of acute choline deficiency in albino weanlings, with mortality of 25/62, or 40%, within 12 days. The supplemented diet was prepared by adding to the test diet choline chloride 500 mg/k, methionine 1 g/k, and inositol 100 mg/k. Twenty-five weanlings receiving this latter diet for 3 weeks showed a normal growth curve, and autopsies revealed no evidence of choline (or other) deficiency. 116 male weanling albino rats of Wistar

TABLE I. Vascular Contractility and Fragility.

| Group | (A) | (B) | (C) | (D) | (E) |
|---|--------------|--------------|--------------|--------------|--------------|
| No. of animals | 68 | 20 | 10 | 7 | 10 |
| Age (days) | 33 | 38 | 88 | 33 | 33 |
| Avg wt (g) | 47.3 | 71.1 | 238 | 45.3 | 55 |
| Mortality (%) | 14 (10) | 0 | 0 | 0 | 0 |
| Epinephrine threshold (million) | 1:57 | 1:12 | 1:9 | 1:70 | 1:10 |
| | S.D. = 24.6 | 2.3 | 1.8 | 21.6 | 1.6 |
| Electrical threshold (volts) | 2.8 | 4.6 | 6.5 | 2.4 | 5 |
| | S.D. = .69 | 2.6 | 2.7 | 2.4 | 2.6 |
| *Rupture threshold arterioles (volts) | 8.3 (44%) | 17 (15%) | † | † | † |
| Venules (volts) | 9.1 (72%) | 16 (30%) | 20 (10%) | 18 (10%) | 20 (20%) |
| Capillaries (volts) | 4.5 (37%) | 7 (10%) | † | † | † |
| Voltage producing venular stasis & duration of stasis | 7 12 min. | 12 4 min. | 15 5 min. | 14 4 min. | 12 5 min. |

* Figure in parenthesis indicates % of animals in which rupture occurred.

† None produced.

strain were separated into 5 diet groups (Table I) as follows: Group A—*Choline deficient*. 68 animals (in groups of 6-10) were fed the deficient diet until signs of deficiency appeared (7-10 days), then were studied as described below. Survivors were given the fully supplemented diet an additional 45 days then the carotid blood pressure was measured by cannulation.

Group B—*Choline supplemented*. 20 animals (in groups of 2-4) were given the fully supplemented diet 7-10 days before examining capillary vessels, as below.

Group C—*Choline deficient hypertensive*. 11 animals. These were fed the deficient diet for 7-12 days until signs of deficiency appeared, then placed on fully supplemented diet for 45 days more to permit possible development of "choline deficiency hypertension" prior to study.

Group D—*Choline supplemented*— "starved," 7 animals. These were pair fed with 7 rats from the deficient group (A) for 7-12 days before examination, to observe possible vascular influences of the reduced dietary intake noted in deficient animals after 3-4 days.

Group E—*Standard laboratory pellet diet*. 10 animals were given the routine diet for 7-10 days prior to study of their peripheral vascular system, to rule out possible influences of the "synthetic" diet itself, even though fully supplemented.

Direct observations of the capillary bed were carried out on all 116 animals by exteriorizing the meso-appendix with Zweifach's method(7). After measuring the threshold responsiveness to topically applied epinephrine, relative reactivity to an electrical stimulus of known strength was determined with silver micro-electrodes and an electronic-type stimulator. By increasing the threshold voltage strength slowly, that necessary to produce rupture of the vessel wall in arterioles, capillaries, and venules could be established. It was also possible to discover the voltage levels necessary to produce intravascular "thrombosis" or stasis, within the venules.

Results. 1. Vascular contractility. The meso-appendiceal arterioles of the acutely choline deficient weanlings (Group A) showed a significantly increased reactivity to both topical epinephrine and electrical stimulation when compared with those on a fully supplemented diet (Group B) and the standard laboratory pellets (Group E), (Table I). This phenomenon cannot be attributed specifically to choline deficiency, however, for it was also observed in Group D receiving a complete diet, but in "starvation" amounts matched with the daily intakes of Group A. Olsen and Deane(8) have described hyperactivity of the zona fasciculata in weanlings during acute choline deficiency, which they attribute to the "alarming" stimulus of inanition. Deane and Shaw(9) had previously described simi-

lar alterations of the zona fasciculata with increased secretion of ketosteroids in rats on a moderate starvation regimen. Groups A and D therefore would be expected to have in common adrenal cortical hyperactivity, presumably due to an alarm response resulting from inanition. The question whether increased arteriolar responsiveness may be related to adrenal hyperfunction is one that is worth further study.

2. *Vascular fragility and venular stasis.* From the data in Table I, it is apparent that arterioles, venules, and capillaries of choline deficient rats are more susceptible to rupture by electrical stimulation than those of any other group, both with respect to the threshold stimulus required to produce rupture and to the frequency with which such damage can be produced. Hemorrhagic phenomena are widespread in acute choline deficiency, for hemorrhages can be noted in the eye, adrenals, heart, and liver, although with less frequency in these organs than in the kidney. During the minor manipulations of the abdominal organs incidental to the studies carried out, intestinal ecchymoses were common in the acutely deficient animals, and were not seen in the others, a gross corroboration of the data presented in the table. Increased fragility of these components of the capillary bed appears then to be specific result of acute choline deficiency in the young rat. Best(10) has recently demonstrated extensive deposition of fat droplets in the intima of vessels in acute choline deficiency. It may be that such a deposition of fat increases the fragility of these vessels. A similar explanation may be advanced for the marked tendency to venular thrombosis among the deficient group; the fat laden vessel wall resists injury poorly. In past investigations of acute choline deficiency in the young rat, most attention has centered upon the most prominent pathological feature, so-called hemorrhagic renal degeneration. The pathogenesis of this process has not yet been completely explained. Hartroft and Best(11,12) have stated that the initial lesion is accumulation of fat droplets in cells of the proximal convoluted tubules of the cortex. According to their observations, there follows swelling of the nephron, with obstruction of

TABLE II. Blood Pressure Determinations in Rats after Acute Choline Deficiency.

| | No. of animals | Avg B.P., mm Hg | S.D. |
|--|----------------|-----------------|------|
| Group (1)—12-18 wk after initial study | 18 | 154 | 22.7 |
| Group (2)—6-12 wk | 14 | 134 | 12.6 |
| Control group—age, 16 wk | 10 | 108 | 7.0 |

the cortical capillary plexus, which in turn leads to ischemia and hemorrhage. Christensen(13,14) has described "massive destruction of the vascular system in the peripheral zone of the cortex" during the acute deficiency stage. It seems likely that the loss of integrity of the vessel wall, particularly of the arterioles, manifested by increased vascular fragility and susceptibility to injury, may be considered an essential factor in the pathogenesis of renal degeneration in acute choline deficiency.

3. *Blood pressure determination.* Table II contains data which illustrate the upward trend of blood pressure with advancing age, in formerly acutely choline-deficient rats. These results are in agreement with the observations of Hartroft and Best. This steady rise in blood pressure is ascribed to the chronic nephropathy resulting from the acute renal injury, perhaps analogous to the progressively higher blood pressure in humans with chronic nephritis. In this study, 46% of the rats became hypertensive within 6 to 18 weeks. It would appear that this procedure could be utilized more widely as a method of producing experimental hypertension, inasmuch as it is very simple and attended by a negligible mortality.

Summary. 1. During the acute phase of choline deficiency in albino weanling rats, arterioles, venules and capillaries of the meso-appendix exhibit a significantly increased fragility, as measured by response to electrical stimulation, in comparison with control groups. There is also a definitely increased sensitivity of arterioles to epinephrine due to inanition. Venular stasis is more rapidly produced, and is of longer duration among choline-deficient animals than in controls. These vascular phenomena disappear upon addition

of choline to the deficient diet. 2. The hypothesis is advanced that these phenomena are reflections of an increased susceptibility to injury of the vessel wall, probably related to deposition of lipid, and that this condition may be an essential factor in the pathogenesis of hemorrhagic renal degeneration. 3. Blood pressure of formerly choline deficient rats tends to rise with time. It is suggested that this method of producing experimental hypertension be used more frequently, because of its simplicity and effectiveness.

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Enzymatic Defect in Metabolism of Bilirubin in Fetal and Newborn Rat. (23719)

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Hyperbilirubinemia and kernicterus can occur in the newborn in the absence of increased hemolysis of the erythrocytes(1-4). The incidence of this nonhemolytic hyperbilirubinemia is particularly high in the premature infant. A low rate of clearance of bilirubin in the liver due to some functional immaturity of that tissue may be responsible for the condition(5). Since bilirubin apparently cannot be cleared by the fetal liver, it has been suggested that this pigment may pass through the placenta and be carried to the mother's liver(6) or may be metabolized by the placental tissue itself(7). Bilirubin is excreted in bile as a conjugate with glucuronic acid(8,9). This conjugation takes place in the liver, to a lesser degree in the kidney(10), and possibly in the stomach and intestine(11). A scheme of the conjugation mechanism for glucuronides has been proposed(12): glucose-1-phosphate + uridine triphosphate \rightarrow uridine diphosphoglucose $\xrightarrow{\text{DPN}}$ uridine diphosphoglucuronic acid (UDPGA) + aglycone \rightarrow aglycone glucuronide. Although conju-

gation of *o*-aminophenol with glucuronic acid could be demonstrated in slices of adult rat liver(13,14), no appreciable conjugation was found in slices of fetal rat liver.

In a previous study using tissue homogenates, we demonstrated a defect in the conjugation of bilirubin in adult rats with constitutional nonhemolytic hyperbilirubinemia(15, 16). In the present study homogenates were again used to investigate the nature of the mechanism for synthesis of bilirubin glucuronide in tissue from prepartum and postpartum rats of various ages.

Methods. The homogenate system used was similar to that described previously(10). Forty micrograms of bilirubin suspended in an albumin solution were added to 100 mg of tissue homogenate in phosphate buffer. One ml of boiled extract of adult liver(17) (extract A) or of fetal liver from animals 5 to 7 days prepartum (extract B) was also added as a source of UDPGA. The mixture was incubated for 45 min. at 37°, then centrifuged in a Servall refrigerated centrifuge

TABLE I. Amounts of Conjugated Bilirubin Produced by Homogenates of Tissue from Adult and Fetal Rats ($\mu\text{g}/100 \text{ mg Wet Wt Tissue}$).

| Exp. No. | Adult | | | | Fetal* | | |
|----------|-------|--------|--------------------|----------|--------|--------|--------------------|
| | Liver | Kidney | Stomach and intes. | Placenta | Liver | Kidney | Stomach and intes. |
| 1 | 6.3 | 2.4 | .1 | .0 | .3 | .0 | .0 |
| 2 | 7.2 | 3.0 | .0 | .0 | .0 | .1 | .0 |
| 3 | 6.7 | 2.2 | .0 | .0 | .2 | .1 | .1 |

* Age of fetuses varied from 5 to 7 days prepartum.

for 5 min. at 10,000 g to remove excess proteins and to stop further enzyme action. "Zero-time" incubations served as the experimental controls. The amount of bilirubin glucuronide produced was determined after centrifugation by measuring the increase in "direct" reacting azo pigment in the supernatant, using the method of Ducci and Watson(18). All determinations were made in duplicate. For assay of β -glucuronidase $2.0 \times 10^{-5} \text{ M}$ phenolphthalein glucuronide was substituted for bilirubin as a substrate and incubated under the conditions described above. The hydrolysis of the phenolphthalein glucuronide was followed by measuring, in alkaline medium at $550 \text{ m}\mu$, the free phenolphthalein released. Homogenates were prepared from the liver of Wistar rats, ranging in age from 10 days prepartum to 16 weeks postpartum. Similar homogenates were prepared of kidney, stomach and gastrointestinal tract from fetal animals 5 to 7 days prepartum, and of placenta from the mother. (A 21-day gestation period was assumed for the purpose of estimating the prepartum age of the animals.) Since the production of bilirubin glucuronide in homogenates is not entirely reproducible

from day to day, parallel determinations of the conjugative activity in tissues from 4-month-old male rats were used as standards for each experiment. These values were taken to represent 100% synthesis.

Results. As seen in Fig. 1, no conjugation of bilirubin with glucuronic acid was detected in the liver from fetal rats up to 4 days prepartum. At about this time conjugative activity was first observed, and by the day of birth it had reached approximately 50% of that found in the liver of adult rats. The conjugative activity continued to increase at a slower rate until it reached the control level by 5 to 6 weeks. The shape of the curve was not markedly affected whether the activity was expressed on the basis of mg wet weight of the tissue or on the basis of μg of nitrogen.

The amounts of conjugated bilirubin produced by homogenates of various tissues from adult and fetal rats are compared in Table I. The conjugative activity in the kidney tissue of the adult rats was about $\frac{1}{3}$ that found in the liver. No activity could be detected in the kidney tissue of the fetal animals. Regardless of the age of the rats, homogenates of stomach, intestine, or placenta* showed no apparent synthesis of bilirubin glucuronide.

Fig. 2 shows the results obtained when fetal liver was used as a source of UDPGA. Enhancement of bilirubin glucuronide synthesis by fetal liver extracts was only about 10% that obtained with extracts from a corresponding amount of adult liver. Addition of fetal liver homogenates from animals 5 to 7 days prepartum to an incubation mixture containing adult liver did not inhibit the bilirubin conjugative activity of the latter tissues (Fig. 2).

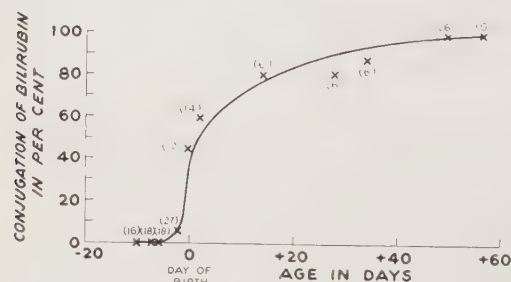


FIG. 1. Conjugation of bilirubin in liver homogenates from rats of various prepartum and postpartum ages. Conjugation of bilirubin in tissue from 4-mo-old adult rats was taken to represent 100%. Figures in parentheses indicate No. of animals.

* Identical results were obtained with homogenates of human placenta.

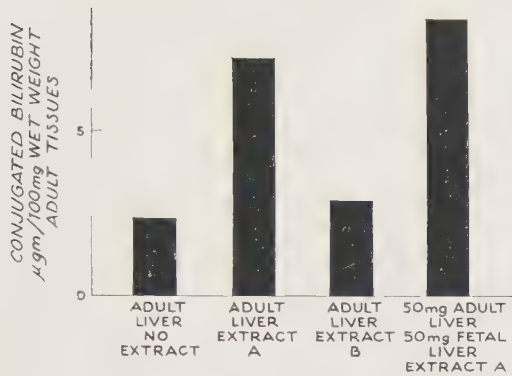


FIG. 2. Effect of homogenates and extracts of fetal liver on conjugation of bilirubin in homogenates of adult liver. Extract A: boiled adult liver, a source of uridine diphosphoglucuronic acid; Extract B: boiled fetal liver.

The addition of 10^{-3} M potassium saccharate to inhibit β -glucuronidase activity failed to influence the conjugative activity in the tissues studied. No hydrolysis of phenolphthalein glucuronide was observed under the conditions of the experiment.

Discussion. Despite the addition of adequate amounts of both bilirubin and UDPGA, no conjugation of bilirubin could be observed in homogenates of liver from fetal rats.[†] This finding supports the hypothesis that the activity of the enzyme responsible for the transfer of the glucuronic moiety to bilirubin (glucuronyl transferase) is defective in the fetal liver. The failure to observe conjugation of bilirubin in this homogenate system does not necessarily establish that glucuronyl transferase itself is absent. Nonetheless, the presence of inhibitors or absence of cofactors in the fetal tissue could not be demonstrated by the mixed homogenate technic.

Not only is glucuronyl transferase activity impaired, but apparently an earlier defect in glucuronide metabolism exists since the levels of UDPGA found in the fetus were only 1/10 that in the adult tissue. By contrast, in constitutional hyperbilirubinemia, another condition in which a defect in glucuronyl transferase activity has been demonstrated(16),

UDPGA levels were normal(19). A number of defects early in the metabolic chain of glucuronide synthesis may exist in the immature liver of the fetus.

The presence of excessive amounts of β -glucuronidase could account for the failure to observe glucuronide production in fetal tissue. This seems improbable since potassium saccharate in amounts capable of inhibiting this enzyme(17) did not influence conjugation. Furthermore, no hydrolysis of phenolphthalein glucuronide was demonstrable.

The defects observed were not specific to fetal liver since conjugation of bilirubin could not be detected in homogenates of fetal kidney. Although Hartiala(14) reported that *o*-aminophenol is rapidly conjugated in slices of stomach and intestine from adult rabbits, we could not demonstrate bilirubin conjugative activity in homogenates of these tissues from adult or fetal rats. It seems unlikely that bilirubin is conjugated by the placenta (7) since no conjugative activity could be detected in homogenates of this tissue.

The finding that glucuronyl transferase activity is impaired in the fetal and newborn rat suggests that a similar lack may be responsible for the hyperbilirubinemia and kernicterus often observed in the premature infant. Although there is good evidence that bilirubin interferes with oxidative phosphorylation *in vitro*(20,21), the toxic effect of elevated bilirubin levels has not been definitely established(22). Steroids, hormones and various phenols(23,24) are normally inactivated and excreted as glucuronides. In the premature infant elevated levels of these substances resulting from impaired glucuronyl transferase activity might be responsible for some of the toxic symptoms observed in association with kernicterus.

Summary. 1. Homogenate preparations of liver and kidney from fetal rats failed to synthesize bilirubin glucuronides. The conjugative activity did not reach normal levels until the animal was 5 to 6 weeks old. 2. The levels of uridine diphosphoglucuronic acid were only 10% of those found in adult tissue. 3. Inhibition of beta glucuronidase with potassium saccharate did not stimulate the conjugation of bilirubin. Phenolphthalein glu-

[†] In an abstract which appeared during preparation of this manuscript, Lathe and Walker reported impairment of glucuronyl transferase activity for bilirubin conjugation in homogenates of human fetal liver (*Biochem. J.*, 1957, v67, 9P).

curonide was not hydrolyzed. 4. No conjugation was observed in homogenates of stomach, intestine or placental tissue. 5. The evidence presented suggests that glucuronyl transferase activity and the production of uridine diphosphoglucuronic acid are impaired in the newborn and the premature animal.

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Renal Clearance of Citric Acid in the Dog.* (23720)

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In alkalosis there is an increased excretion of citric acid(1). This is a report of the effect of administration of bicarbonate and citric acid or citrate upon renal clearance of citric acid.

Methods. All clearances were determined on trained conscious female dogs at least 15 hours after removal of food. With one exception the dogs weighed 16 to 31 kg. Catheterization and bladder washing were used. Generally, clearance periods began about 45 minutes after oral administration of Na or KHCO_3 and creatinine. In other cases, the periods were concurrent with intravenous in-

jection of these compounds. Citric acid was determined by the method of Perlman *et al.* (2). Creatinine was determined with alkaline picrate. A total of 126 clearance periods were done on 42 different days on 10 dogs. Typical data are in the tables. Citric acid concentration in an ultrafiltrate of plasma, which was in equilibrium with 5.5% CO_2 , was identical with that in plasma water. The amount of citric acid filtered was calculated as the product of creatinine clearance (C_{cr}) and plasma concentration of citric acid.

Results. Clearance of citric acid (C_{ca}) in the controls was less than 1 ml/min. in 28 of 40 determinations on 9 dogs. Of the 12 clearances greater than 1 ml/min., 7 were from dog C. The maximum for this dog was 15.1 ml/

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TABLE I. Citric Acid Clearance after Bicarbonate.

| Dog | Procedure | | Citric acid | | Tubular absorption | | Creatinine clearance, ml/min. |
|-----|-------------------------------------|-----|-------------------|--------------------|---------------------------|------------------|-------------------------------|
| | | | Plasma, mg/100 ml | Clearance, ml/min. | T _{ca} , mg/min. | % of filtered ca | |
| S | Control | (1) | 4.25 | .37 | 2.21 | 99.3 | 52.3 |
| | 10 g NaHCO ₃ | (2) | 4.25 | 3.14 | 1.78 | 93.0 | 45.1 |
| | 5 g " | (3) | 4.0 | 2.46 | 1.89 | 95.1 | 49.7 |
| | Control | (1) | 3.87 | .83 | 1.36 | 97.7 | 36.0 |
| | 15 g KHCO ₃ | (2) | 4.50 | 8.07 | 1.55 | 81.9 | 42.4 |
| | | (3) | 5.50 | 6.98 | 1.86 | 82.9 | 40.8 |
| | | (4) | 5.00 | 6.06 | 1.87 | 85.7 | 43.5 |
| | | | | | | | |
| N | Control | (1) | 5.25 | .44 | 2.96 | 99.2 | 56.8 |
| | 10 g NaHCO ₃ | | | | | | |
| | 5 g " | (2) | 5.75 | 2.89 | 2.56 | 93.9 | 47.5 |
| | Control | (1) | 5.62 | .86 | 3.96 | 98.8 | 71.4 |
| | 4 g NaHCO ₃ i.v. | (2) | 5.37 | 5.52 | 3.17 | 91.4 | 64.5 |
| | | (3) | 6.12 | 5.33 | 3.75 | 92.0 | 66.6 |
| Ch | Control | (1) | 4.45 | .92 | 3.79 | 98.9 | 86.1 |
| | 20 g NaHCO ₃ | (2) | 4.45 | 9.58 | 2.97 | 81.5 | 76.4 |
| | | (3) | 4.72 | 7.23 | 3.19 | 90.3 | 75.4 |
| C | Control | (1) | 5.92 | .71 | 4.05 | 99.1 | 75.4 |
| | 15 g NaHCO ₃ | (2) | 6.00 | 19.63 | 2.50 | 68.0 | 61.3 |
| | 5 g " 40 min. later | (3) | 5.65 | 10.45 | 2.93 | 83.2 | 62.3 |
| | Control | (1) | 6.17 | 9.56 | 4.44 | 88.2 | 81.5 |
| | 15 g NaHCO ₃ | (2) | 5.10 | 26.75 | 4.45 | 76.4 | 114.0 |
| | | (3) | 6.12 | 15.88 | 4.96 | 83.6 | 97.0 |
| B | Control | (1) | 4.03 | 1.03 | 1.81 | 92.7 | 45.9 |
| | 15 g NaHCO ₃ | (2) | 4.82 | 4.50 | 2.01 | 90.3 | 46.1 |
| | | (3) | 4.82 | 5.64 | 1.68 | 86.1 | 40.4 |
| Pe | Control | (1) | 2.90 | .72 | 1.59 | 98.7 | 55.7 |
| | .35 g NaHCO ₃ /min. i.v. | (2) | 4.30 | 18.75 | 1.75 | 68.4 | 59.4 |
| | No infusion | (3) | 3.30 | 21.40 | 1.53 | 53.9 | 46.4 |
| | " " | (4) | 5.20 | 11.50 | 2.43 | 70.4 | 46.7 |

min. The ratio of C_{ca} to C_{cr} for all controls was less than 0.03 with 6 exceptions. Administration of NaHCO_3 or KHCO_3 was followed by a marked increase in the C_{ca} in 24 of 27 experiments (Table I). These 2 salts appeared to be equally effective. The maximum C_{ca} in 6 experiments was in the range 1.5 to 3.0 ml/min.; in 7 experiments it was 5-10 and in 9 experiments it was above 10. In the 24 cases of the rise, ratio of C_{ca} to C_{cr} rose more than 4-fold with 2 exceptions. Furthermore, the rise in C_{ca} in 7 experiments on 5 of 9 dogs occurred when the calculated filtered load of citric acid was less than during the immediately preceding control period and of course, the percentage of filtered citric acid absorbed by the tubules markedly decreased. In 2 other experiments, involving an additional dog, the increase in filtered load was insignificant. Therefore, in 9 experiments on 6 dogs, the rise in C_{ca} after BHCO_3 was

clearly due to less tubular absorption of citrate.

One way by which alkalosis might result in the tubular absorption of a lesser fraction of the filtered citric acid is that of competition by other organic acids for the same cellular absorptive mechanism. Citric acid has been found to account for only about 50% of the total organic acids in the urine during alkalosis(3). In the cases of increased filtered load, the extra load was due in 4 experiments to a rise in plasma citrate, in 5 experiments to an increase in filtration and in the others to a combination of these 2 factors.

Administration of citric acid or citrate was followed by an increase in C_{ca} in 12 of the 14 experiments on 5 dogs (Table II). The ratio of C_{ca} to C_{cr} increased 2 to 3.5 fold in 7 experiments and more in the others. With only one exception, the rise in C_{ca} was associated with a marked increase in the filtered

TABLE II. Citric Acid Clearance with Exogenous Citric Acid.

| Dog | Procedure | | Citric acid | | Tubular absorption | | Creatinine clearance, ml/min. |
|-----|------------------|-----|-------------------|--------------------|--------------------|------------------|-------------------------------|
| | | | Plasma, mg/100 ml | Clearance, ml/min. | T_{ca} , mg/min. | % of filtered ca | |
| S | Control | (1) | 4.50 | .82 | 1.87 | 98.1 | 42.4 |
| | 12 g citric acid | (2) | 14.10 | 2.86 | 5.72 | 93.4 | 43.4 |
| | | (3) | 14.30 | 2.91 | 5.86 | 93.4 | 43.9 |
| N | Control | (1) | 4.30 | .54 | 2.03 | 98.8 | 47.7 |
| | 10 g citric acid | (2) | 10.50 | 1.63 | 6.06 | 97.2 | 59.3 |
| | | (3) | 9.75 | 1.60 | 5.54 | 97.2 | 58.4 |
| C | Control | (1) | 6.25 | 6.36 | 3.32 | 89.3 | 59.4 |
| | 8 g citric acid | (2) | 11.50 | 14.20 | 6.81 | 80.6 | 73.4 |
| | | (3) | 7.25 | 17.59 | 3.51 | 73.3 | 66.0 |
| B | Control | (1) | 5.62 | .28 | 2.89 | 99.4 | 51.7 |
| | 8 g citric acid | (2) | 8.80 | 7.41 | 5.20 | 87.5 | 64.3 |
| | | (3) | 8.75 | 5.32 | 4.99 | 91.5 | 62.4 |
| Y | Control | (1) | 4.08 | 1.23 | 2.40 | 98.0 | 60.0 |
| | 15 g citric acid | (2) | 13.85 | 3.78 | 5.30 | 95.0 | 76.1 |
| | | (3) | 13.18 | 2.72 | 5.83 | 96.2 | 72.2 |

load of citric acid, chiefly due to a rise in plasma citrate. Therefore, the increase in C_{ca} in these cases was probably due to excessive loading of the cellular mechanism for citrate absorption.

In Table III it is seen that the rate of tubular citrate absorption (T_{ca}) after $BHCO_3$ approximates that in the control but is much higher after ingestion of citric acid. Nevertheless, the C_{ca} was least in the control and in 13 cases higher in the same dog after $BHCO_3$ than after exogenous citrate. This comparison also indicates that $BHCO_3$ has produced changes in tubular absorption of citrate.

The data obtained after administration of citric acid show that if there is a maximal rate of absorption, it is extremely variable. For example, in dog N T_{ca} ranged from 3.7 to 9.6 mg/min. The toxicity(4) of citrate prohibited elevation of its plasma concentration sufficiently to ascertain if there was a maximal rate of tubular absorption similar to that for glucose. One dog developed tetany and died

although calcium was given intravenously.

With regard to the specificity of the effect of $BHCO_3$ on the tubular reabsorption of citric acid, its basic or acidic equivalents of disodium phosphate or ammonium chloride respectively were found to be ineffective. However, in one test NaCl effected a small rise in C_{ca} and T_{ca} . Calcium lactate (2 g) or gluconate (2.75 g) given intravenously were ineffective.

In 2 experiments, after C_{ca} was elevated by oral administration of citric acid, intravenous infusion of ascorbic acid in doses calculated to saturate its tubular mechanism of absorption(5) slightly lowered C_{ca} and the ratio of C_{ca}/C_{cr} . Presumably, these 2 acids are not absorbed by the same mechanism.

In acute alkalosis there is increased excretion of potassium(6,7). In 3 dogs, although the clearance of potassium was elevated to 68 to 105% of the C_{cr} by chronic daily oral dosage of KCl and intravenous infusion of KCl, C_{ca} remained at the control level. Therefore, although both C_{ca} and C_K are elevated in alkalosis they are not presumably causally related.

Summary. The clearance of citric acid under control conditions was generally less than 1 ml/min., being less than 3% of the filtration clearance. Its clearance was markedly elevated after administration of $BHCO_3$, due chiefly to less absorption by the renal tubules. Administration of citric acid was

TABLE III. Mean Values for Tubular Absorption of Citric Acid (mg/min.).

| Dog | Control | After administration of | |
|-----|------------------|-------------------------|------------------|
| | | $BHCO_3$ | Citric acid |
| S | 1.725 \pm .43* | 1.876 \pm .48 | 3.640 \pm 1.96 |
| N | 2.198 \pm 1.04 | 2.437 \pm .75 | 6.266 \pm 1.63 |
| C | 3.299 \pm .85 | 3.249 \pm 1.02 | 5.684 \pm 1.25 |
| B | 1.912 \pm .60 | 2.532 \pm .67 | 5.473 \pm 2.41 |

* Stand. dev.

followed by a comparable elevation of clearance accompanied by an increase both in the filtered load and the amount absorbed by the tubules. Data indicated that the tubular absorption of citric acid was not reduced by ascorbic acid. Administration of potassium did not increase the citrate clearance.

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Effect of Histamine and Histamine Liberator, Compound 48/80, on Blood Glucose.* (23721)

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The relationship between adrenal glands and the action of histamine was first revealed by Dale(1) who showed that small doses of histamine produced dilatation of the sensitized pupil of the cat similar to the dilatation produced by small doses of epinephrine. Burn and Dale(2) established that the secondary rise in blood pressure in the cat following injection of histamine was abolished by removal of the adrenals. Histamine injected in small amounts (1 μ g or less) into the arterial blood supplying the adrenal gland of the cat is known to liberate epinephrine as judged by pressor responses and retraction of the nictitating membrane(3,4). Another more sensitive index of the action of injected or liberated epinephrine is the hyperglycemia resulting from the known glycogenolytic activity of this substance. Histamine injected intravenously into dogs, rabbits and rats(5,6,7) has been reported to produce hyperglycemia which is presumably due to liberated epinephrine. In some of the studies involving hyperglycemic responses in the dog and rabbit

rather large doses of histamine have been used, and the rise in blood glucose could result from direct stimulation of the adrenal medulla by injected histamine and/or from reflex sympathetic activity coincident with severe shock. Experiments were therefore made in unanesthetized dogs and rabbits using several doses of histamine to determine whether a graded hyperglycemia could be demonstrated in the absence of moderate or severe grades of shock. In another series of experiments, compound 48/80 which is designated as a potent liberator of endogenous histamine(8), was employed in a similar manner for the purpose of comparing the 2 substances with respect to possible hyperglycemic activity.

Methods. In the histamine series, trained male albino rabbits weighing 3 to 4 kg were fasted 18-20 hours. Blood samples were taken from each animal 3 to 5 minutes before the injection of saline or histamine diphosphate and again after 15 to 60 minutes. Controls received physiological saline, while test animals received 0.1, 0.15 and 0.2 mg/kg of histamine in terms of the base. Higher doses of 0.25 and 0.3 mg/kg proved to be lethal in several instances. Each control and experimental group comprised 9 to 11 animals. In addition, each of 4 trained mongrel dogs, fasted approximately 20 hours, was injected

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TABLE I. Influence of Histamine and Compound 48/80 on Blood Glucose (mg %) of Trained Rabbits.

| Dose, mg/kg | | Min. after inj. | | | |
|---------------------|----------------|-----------------|---------------|----------------|----------------|
| intrav. | Preinj. level | 15 | 20 | 30 | 60 |
| I. Histamine (base) | | | | | |
| .1 | 90 \pm 2.2* | 96 \pm 4.3 | 94 \pm 4.7 | 90 \pm 4.1 | 99 \pm 6.6 |
| .15 | 94 \pm 2.1 | 98 \pm 5.5 | 99 \pm 7.3 | 97 \pm 4.7 | 101 \pm 4.0 |
| .2 | 93 \pm 2.0 | 100 \pm 6.1 | 105 \pm 6.1 | 110 \pm 6.7† | 106 \pm 4.8† |
| II. Compound 48/80 | | | | | |
| .5 | ‡115 \pm 3.7 | 120 \pm 8.5 | 119 \pm 8.7 | 116 \pm 10.2 | 115 \pm 11.0 |
| | §120 | 152 | 154 | 156 | 162 |
| | §123 | 134 | 134 | 132 | 121 |

* Each value represents mean \pm stand. error of 9-11 animals.

† P < .05 indicating a statistically significant change.

‡ Each value represents mean \pm stand. error of 6 animals.

§ Individual values for 2 animals in this group showing toxic symptoms.

twice on different occasions with physiological saline and twice with each of 3 doses of histamine equivalent to 0.05, 0.1 and 0.2 mg/kg of the base. Blood samples were taken 3 minutes before and at 10 to 60 minutes after injection. In experiments with compound 48/80, trained male albino rabbits were treated as in the histamine group. Each control and test group comprised 5-6 animals, controls receiving saline and test groups 0.3 or 0.5 mg/kg of 48/80. Trained mongrel dogs also received saline or 48/80 (0.1 and 0.5 mg/kg), each control or experimental group comprising 6 to 7 animals. Blood samples were collected as described above. Injections were made intravenously during 10 seconds in volume of 0.5 to 1.0 ml. Blood glucose was determined by semi-micro technic of Folin-Malmros(9).

Results. Histamine. In rabbits we demonstrated a graded hyperglycemia following histamine. At highest dose of 0.2 mg/kg many animals manifested moderate to severe grades of shock and in these cases a moderate hyperglycemic response was evident (Table I). In dogs, a dose of 0.2 mg/kg histamine base caused a significant increase in blood sugar (Table II). This dose induced urination, salivation, lacrimation and tenesmus in each dog. Two animals collapsed and exhibited labored respiration. Lower doses of 0.05 and 0.1 mg/kg produced milder responses but failed to raise the blood sugar level.

Compound 48/80. In 6 rabbits, a relatively high dose of this compound (0.5 mg/kg) produced no significant change in blood glucose except for a slight hyperglycemia

(Table I) in 2 animals manifesting toxic symptoms of mild dyspnea, cyanosis and thrashing about. A dose of 0.3 mg/kg in 6 other animals produced no symptoms and no change in blood glucose. Six dogs which received 0.1 mg/kg, exhibited no significant changes in blood sugar. The animals reacted with shaking of head, rubbing head against floor and scratching of neck, for approximately 20 minutes. In 7 dogs receiving 0.5 mg/kg, symptoms consisted of shaking of head, ataxia, urination, defecation, and complete prostration. The animals usually recovered but were still unsteady at the end of the hour. A moderate fall in blood glucose was observed at 10 and 20 minutes after injection. At 60 minutes the level had increased 11 mg % above the control value (Table II). Thus compound 48/80 induced a slight transient hypoglycemia in the dog at a time when histamine shock induced a hyperglycemia.

Discussion. Histamine in minute quantities has direct epinephrine releasing action on the cat adrenal gland(1-4). This finding may be construed to mean that hyperglycemia previously reported to occur in dogs and rabbits after intravenous injections of histamine may also be due to a direct effect of histamine on the adrenal medulla. In our experiments small doses of histamine which produced mild symptoms in the dog and rabbit failed to raise the blood sugar level, whereas a moderate degree of hyperglycemia was induced when the dose was high enough to produce toxic effects.

The studies with 48/80 in rabbits revealed that the larger dose of 0.5 mg/kg was mildly toxic for 2 of 6 animals and in these instances

TABLE II. Comparison of Effects of Histamine and Compound 48/80 on Blood Sugar (mg %) of Trained Dogs.

| Drug | Dose, mg/kg intrav. | Preinj. level | Min. after inj. | | |
|-----------|------------------------|---------------|-----------------|---------------|----------------|
| | | | 10 | 20 | 60 |
| Histamine | .05 | 79 \pm 2.6 | 84 \pm 4.3 | 81 \pm 3.3 | 84 \pm 4.2 |
| | .1 | 81 \pm 3.8 | 87 \pm 6.2 | 86 \pm 6.2 | 84 \pm 6.0 |
| | .2 | 82 \pm 3.5 | 92 \pm 5.7* | 98 \pm 5.5* | 91 \pm 5.5* |
| 48/80 | .1 | 87 \pm 3.3 | 84 \pm 5.5 | 83 \pm 5.6 | 84 \pm 3.4 |
| | .5 | 89 \pm 2.7 | 75 \pm 3.1* | 82 \pm 2.3* | 100 \pm 4.8* |

Each value for histamine group is mean \pm stand. error of 8 observations in 4 animals; for 48/80, mean \pm stand. error of 6 to 7 animals (one observation/animal).

* P value $< .05$.

an actual hyperglycemia developed. However, analysis of the results for the whole group receiving this dose showed no significant changes. There was an indication therefore that compound 48/80, like histamine, did not induce hyperglycemia in rabbits unless it produced symptoms of toxicity.

The effect of 48/80 in dogs presented an entirely different picture. During histamine shock, a marked hyperglycemia develops 20 to 30 minutes after injection(5). In our studies, it was found that a moderate increase in blood glucose was manifest 10 and 20 minutes following injection of 0.2 mg/kg of histamine base. When compound 48/80 was administered at a dose level (0.5 mg/kg) capable of producing many severe symptoms including prostration in 6 of 7 animals, it induced, instead of a rise in blood sugar, a moderate hypoglycemia in 10 minutes during height of symptoms, still present but less pronounced in 20 minutes. A mild hyperglycemia was then observed at the end of an hour. These developments are the reverse of what would be expected from a potent histamine liberator especially in the dog in which compound 48/80 is known to release large amounts of histamine almost immediately. Since the effects of this compound on blood glucose are not consistent with those following histamine shock, it appears that 48/80 possesses some transient hypoglycemic activity in the dog unrelated to release of endogenous histamine. Such activity is apparently of sufficient magnitude to override any hyperglycemic response from reflex sympathetic activity called into play by the shock-like state induced by 48/80. The secondary mild hyperglycemia which was observed at the end

of an hour may be a late manifestation of epinephrine release.

Summary. It was not possible to induce hyperglycemia in dogs and rabbits with non-toxic doses of histamine injected intravenously, whereas a moderate hyperglycemia occurred in both species when the dose was high enough to produce toxic effects; epinephrine release and glycogenolysis probably resulted from reflex sympathetic activity consequent to the shock induced. Compound 48/80 also did not induce hyperglycemia in rabbits except in those showing toxic symptoms. In the dog, a dose of 48/80 capable of producing severe symptoms, including prostration, caused a moderate fall in blood glucose at a time when histamine shock produced a hyperglycemia. Therefore, compound 48/80 exerted a transient hypoglycemic action in dogs which appears to be unrelated to histamine release.

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High Susceptibility of 1 to 14 Days Old C3H Mice to "Passage A" Leukemic Filtrates.* (23722)

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In 1951 it was observed in our laboratory that mouse leukemia could be transmitted by cell-free filtrates to newborn mice of a susceptible strain(1,2). This observation was recently confirmed by Woolley(3), Furth(4), Dulaney(5), Hays(6), and their associates. Among the extracts prepared from Ak donors with spontaneous leukemia, some were highly active, others less potent, on inoculation tests(7,8.) Using one of the more active extracts, and passing it through several successive cell-free inoculations of newborn hosts, it was possible to obtain a highly potent agent designated "passage A"(8). Filtered extracts prepared from this leukemic passage strain, inoculated into less than 16 hours old C3H mice of the Bittner substrain(9), induced acute lymphatic leukemia in from 40 to 100% of the inoculated mice, and in some instances parotid gland tumors, after a latency period varying from 2½ to 3½ months(8). After several consecutive passages through newborn hosts, an attempt was made to determine whether this highly potent passage agent might now also induce leukemia when inoculated into suckling mice older than 16 hours. In contrast to previous experience, C3H mice 1 to 14 days old were found highly susceptible to inoculation of the leukemic filtrates; young adult C3H mice were also susceptible, though to a considerably lesser extent.

Methods. *Preparation of leukemic extracts.* C3H mice which developed advanced leukemia at 2½ to 3½ months of age as a result of inoculation, when newborn, with filtrates prepared from the 8th, 9th, or 10th cell-free passages, were used as donors. Parts of liver, spleen, mediastinal and mesenteric tumors, and peripheral lymph nodes, were removed aseptically, weighed, ground in a mortar by hand with chilled, sterile physiological saline

added to obtain 20%, and in a few instances 10% concentration. Following centrifugation at 0°C, first at 3000 rpm (1400 x g) for 15 minutes, then at 9500 rpm (7000 x g) for 5 minutes, the final supernate (10 to 12 ml) was mixed with 0.5 ml of 1:2000 dilution of fresh broth culture of *E. coli*, and passed through Sela's porosity 02 porcelain filter candles under vacuum pressure(10). In each instance the filtrate was checked and found to be sterile as evidenced by inoculation of tryptose broth media, thus indicating that the filter candle retained *E. coli*. The filtered leukemic extracts were immediately placed in sterile glass tubes immersed in larger containers filled with ice cubes. Kept at 0°C, most of the extracts were used within 24 hours, none later than after 48 hours. *Test animals.* All mice used for inoculation, except when otherwise stated, were of the C3H, or foster nursed C3H(f) lines, both of Bittner substrain(9). The sexes were separated at weaning time. Those mice that died when less than 6 weeks old, were not included in the tabulation; this concerns particularly the mortality occurring among newborn mice within hours after inoculation. The incidence of spontaneous leukemia, or lymphosarcomas, in untreated mice of our colonies of C3H or C3H(f) mice does not exceed 0.5%, and occurs in mice usually over 15 months of age.

Results. *Inoculation of filtrates into newborn mice less than 16 hours old.* Filtered extracts prepared from passage 8th, 9th and 10th donors, were inoculated into newborn, less than 16 hours old C3H mice (passage 8th refers to inoculations of extracts prepared from passage 7th donors, passage 9th to inoculations from passage 8th donors, etc.) Of 310 mice, 175 (56%) developed leukemia, and 24 (8%) developed parotid gland carcinomas, at 3.9 months average age. Centrifuged (7000 x g) extracts were more potent than filtrates, 40 mice out of 44 inoculated

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TABLE I. Results of Inoculation of Centrifuged or Filtered Leukemic Passage Extracts into Newborn, Less than 16-Hr-Old C3H Mice.

| Passage No. | Extract inoc.* | No. mice inoc.* | No. mice dev. leuk. | Leuk. inc., % | No. mice dev. parot tum | Parot tum inc., % | Total dev. leuk. or parot tum | Total leuk. & parot tum inc., % | Avg age leuk. or parot tum dev., mo |
|-------------|----------------|-----------------|---------------------|---------------|-------------------------|-------------------|-------------------------------|---------------------------------|-------------------------------------|
| 8 | Fil | 64 | 23 | 36 | 15 | 23 | 38 | 59 | 4 |
| | Ctr | 32 | 28 | 88 | 1 | 3 | 29 | 91 | 3.5 |
| 9† | Fil | 120 | 73 | 61 | 1 | 1 | 74 | 62 | 4 |
| | Ctr | 12 | 12 | 100 | | | | | 3.5 |
| 10† | Fil | 126 | 79 | 63 | 8 | 6 | 87 | 69 | 3.6 |
| Total | Fil | 310 | 175‡ | 56 | 24§ | 8 | 199 | 64 | 3.9 |
| | Ctr | 44 | 40 | 91 | 1 | 2 | 41 | 93 | 3.5 |

* Fil = Selas 02 filtrate. Ctr = 7000 x g supernate. All inoculations subcut. Those mice that died when less than 6 wk old are not included in tabulation.

† These experiments are still in progress; avg age of surviving mice, still in good health, is 7 and 5 mo for passage 9th and 10th respectively.

‡ 2 mice developed leukemia and parotid tumors.

§ 2 " " parotid tumors and medullary adrenal tumors.

developing leukemia (91%), and 1 parotid gland tumors (2%) at 3.5 months average age (Table I).

Inoculation of filtrates into suckling mice 16 hours to 21 days old. When suckling mice more than 16 hours old, and up to 14 days of age were inoculated with the filtered leukemic extracts (Table II), 243 out of 304 (80%) developed leukemia at average ages varying from 3.3 to 3.7 months, some as early as 2 months after inoculation. None developed parotid tumors. The incidence of leukemia was higher in mice inoculated intraperitoneally (89%) as compared with those inoculated subcutaneously (69%). Of 28 mice inoculated when more than 14, and up to 21 days of age, only 9 (32%) developed leukemia; this incidence may, however, increase since some of the mice in this group are now only 4 months old.

Inoculation of filtrates into adult mice. The leukemic filtrates were inoculated intraperitoneally or intracranially into 122 young adult C3H mice (average age at inoculation 51 days) and, as a result, 45 of them (37%) developed leukemia at an average age of 8.8 months. None developed parotid tumors (Table III).

Transplantation of leukemic cells from passage 8th, 9th and 10th donors. In 10 experiments, leukemic cell suspensions of 20% concentration were prepared from 8th, 9th and 10th passage donors (in which leukemia re-

sulted from inoculation of filtrates); these cell suspensions were then injected intraperitoneally (0.25 to 0.5 ml) into young adult (2 months old) C3H and Ak mice. All 17 inoculated C3H mice, and 16 of 18 inoculated Ak mice developed leukemia after average latency of 17 and 61 days respectively.

Inoculation of leukemic filtrates into newborn C3H mice of Andervont subline. Young, adult C3H males and females were recently obtained from Dr. H. B. Andervont, Nat. Cancer Inst. These mice were mated in our laboratory, and their newborn offspring were inoculated at average age of 10 hours with leukemic filtrates (Selas 02) prepared from passage 8th, 9th and 10th donors. Of 33 mice only 4 (12%) developed leukemia at 2½, 3, 4, and 4½ months respectively; none developed parotid tumors. In simultaneous experiments, the same filtrates were inoculated into 25 less than 16 hours old C3H mice of the Bittner substrain, and 14 (56%) developed leukemia at average age of 2.8 months. Although not yet completed, these results are essentially similar to those previously reported(9).

Discussion. When in previous experiments cell-free extracts prepared from spontaneous Ak leukemia were inoculated into suckling C3H mice 2 to 12 days old, some of them developed leukemia, but not before 14.5 months of age(2). The potency of the leukemic agent increased through serial cell-free pas-

TABLE II. Results of Inoculation of Filtered Leukemic Passage Extracts* into Suckling C3H Mice 16 Hours to 21 Days Old. None developed parotid tumors.

| Age at inoc., days | Route of inoc.† | No. of mice inoc.§ | No. of mice dev. leuk. | Leuk. inc., % | Avg age leuk. dev., mo |
|-----------------------|----------------------|--------------------|------------------------|---------------|------------------------|
| 16 hr <1† | Subcut. Intraper. | 34 | 21 | 62 | 3.3 |
| | | 3 | 3 | 100 | |
| | | 37 | 24 | 65 | |
| 1 < 2 | Subcut. Intraper. | 54 | 40 | 74 | 3.6 |
| | | 13 | 13 | 100 | |
| | | 67 | 53 | 79 | |
| 2 < 4 | Subcut. Intraper. | 38 | 26 | 68 | 3.3 |
| | | 34 | 31 | 91 | |
| | | 72 | 57 | 79 | |
| 4 < 7 | Subcut. Intraper. | 8 | 5 | 62 | 3.4 |
| | | 56 | 49 | 87 | |
| | | 64 | 54 | 84 | |
| 7 <10 | Subcut. Intraper. | 3 | 3 | 100 | 3.6 |
| | | 26 | 25 | 96 | |
| | | 29 | 28 | 97 | |
| 10 <14 | Intraper. | 35 | 27 | 77 | 3.7 |
| Total: 16 hr <14 days | Subcut. Intraper. | 137 | 95 | 69 | |
| | | 167 | 148 | 89 | |
| | | 304 | 243 | 80 | |
| 14 <21 | Intraper. | 28 | 9 | 32 | |

* All extracts were Selas 02 filtrates prepared from passage 8th, 9th and 10th.

† More than 16 hr, and up to 1 day old.

‡ Subcut. = subcutaneous; Intraper. = intraperitoneal.

§ Those mice that died when less than 6 wk old are not included in tabulation.

sage; the agent now induces leukemia after a latency of $2\frac{1}{2}$ to 4 months when inoculated into suckling mice 1 to 14 days old. Additional experiments are needed, however, to determine whether at least some of the more potent extracts prepared from Ak mice with spontaneous leukemia, may be more active than previously suspected, when inoculated into suckling C3H mice less than 14 days old.

Serial, cell-free passage of the leukemic agent might have increased its potency for the Bittner substrain of the C3H line, increasing incidence of induced leukemia, shortening latency time, and extending age range of susceptibility of the recipient hosts. The ability, however, of the agent to infect recipient hosts of certain genetically distinct inbred lines, did not change materially. A subline of the C3H strain, maintained in Dr. Ander-vont's laboratory at the National Cancer Institute(9), remained relatively resistant to the passage A leukemic strain.

In previous experiments, when leukemia was induced in C3H mice with Ak leukemic filtrates, leukemic cell suspensions prepared from C3H donors could be readily transplanted to adult C3H hosts, but only rarely to adult mice of the Ak line(11,7,3). It is quite interesting, therefore, that C3H leukemia induced with the potent passage filtrates, could now be transplanted by cell-graft to adult mice of both, C3H and Ak inbred lines(4); the latency period, however, was considerably longer in Ak mice inoculated with the C3H leukemic cells (61 days) as compared with C3H recipients (17 days).

The fact that inoculation of passage A leukemic filtrates into C3H mice more than 16 hours old induced leukemia, but not parotid gland tumors, was puzzling. Of 304 mice inoculated when more than 16 hours, but less than 14 days old, 80% developed leukemia, none parotid tumors (Table II). The same filtrates inoculated into 310 mice less than 16 hours old induced either leukemia (56%) or

TABLE III. Results of Inoculation of Leukemic Passage Filtrates* into Adult C3H Mice,†

| Route of inoc.‡ | No. of mice inoc. | No. of mice dev. leuk. | Leuk. inc., % | No. of mice dev. parotid tumor | Avg age leuk. dev., mo | Avg latency, mo |
|-----------------|-------------------|------------------------|---------------|--------------------------------|------------------------|-----------------|
| Intraper. | 94 | 35 | 37 | 0 | | |
| Intracran. | 28 | 10 | 36 | 0 | | |
| Total | 122§ | 45 | 37 | 0 | 8.8 | 7 |

* All extracts were Selas 02 filtrates. Each filtrate was prepared from a different donor.

† 86 were C3H or C3H(f) males; 28 were C3H and 8 C3H(f) females.

‡ Intraper. = intraperitoneal (0.5 to 1.0 ml). Intracran. = intracranial (0.1 to 0.15 ml). Avg age at inoculation, 51 days.

§ 38 mice still alive and well at 12.5 mo avg age. 39 died with no signs of leukemia at 14 mo avg age.

parotid tumors (8%), occasionally both (Table I). Similarly, leukemia (37%) but not parotid tumors developed following inoculation of the filtrates into adult C3H mice (Table III). The striking difference in susceptibility to the induction of parotid tumors, depending on the age of the recipient host at the time of inoculation, may be consistent with the assumption previously expressed that the leukemic agent, and that causing parotid gland tumors, are distinct, though possibly related(10,7).

Previous attempts to transmit leukemia into adult C3H mice with filtrates prepared from initial passages, were seldom successful (8). It was surprising, therefore, to find that filtrates prepared from the more recent passages induced leukemia in 45 out of 122 C3H mice inoculated when approximately 6 to 8 weeks old. Adult hosts were, however, less susceptible than 1 to 14 days old suckling mice; furthermore, the disease appeared late, at 8.8 months average age (Table III). Our passage A leukemic agent is therefore considerably less pathogenic for adult hosts than that developed from Ehrlich ascites carcinoma in Swiss mice by Friend(12).

It is of interest that centrifuged extracts were more potent than filtrates (Table I), and that the intraperitoneal route of inoculation was more effective than subcutaneous (Table II).

The fact that passage A leukemic filtrates were found to be highly active when inoculated into few-days-old C3H mice is of practical value. It may no longer be necessary to prepare for inoculation extracts only when female mice in advanced pregnancy are available, and then check such mice every few

hours for litters, in order to inoculate newborn mice possibly within hours after birth. Several litters can now be accumulated in preparing an experiment, and the extract then made and injected at once into several groups of susceptible suckling mice. Furthermore, mortality resulting from inoculating more than 1-day-old suckling mice is practically nil, even when the more sensitive intraperitoneal route is used, whereas mortality is often very high when newborn mice, less than 16 hours old, are inoculated. Further observations are needed to determine whether suckling mice less than 14 days old are uniformly susceptible to inoculation of leukemic filtrates, and whether there are differences in susceptibility between newborn (less than 16 hours old) mice, and those 2, 5 or 10 days old. It is quite possible that suckling C3H mice 2 to 10, and perhaps even up to 14, days old, may be more susceptible to the leukemogenic action of the passage filtrates than newborn, less than 16 hours old mice.

In studies on chicken leukosis(13-16) and also on Rous sarcoma(17), the observation has long been made that among the susceptible recipient hosts, very young animals were more sensitive to inoculation of the cell-free extracts. There appears to be an optimum age for inoculation of the filtrates, which is different for different forms of chicken leukosis (13-15) and also for different strains of chickens in the case of Rous sarcoma(17).

Summary. 1. After 7 consecutive passages through newborn hosts, passage A leukemic filtrates could be inoculated successfully into suckling C3H mice 1 to 14 days old, inducing leukemia, but not parotid tumors, in 62% to 100% of the inoculated mice at 3.3 to 3.7

months average age. 2. The incidence of leukemia was higher in mice inoculated intraperitoneally (89%) than in those injected subcutaneously (69%). 3. Of 122 young, adult C3H mice inoculated intraperitoneally, or intracranially with leukemic filtrates, 37% developed leukemia (none parotid tumors) at 8.8 months average age. 4. Of 310 newborn C3H mice inoculated when less than 16 hours old with the same leukemic filtrates, 64% developed either leukemia (56%), or parotid tumors (8%), at 3.8 months average age. Two mice in this group developed both leukemia and parotid tumors.

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Plasma Heparin Levels in Man Following Intravenous Fat Emulsions. (23723)

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Clearing of alimentary lipemia(1) and decrease in serum low density lipoproteins(2) following injection of heparin have raised the question of the possible physiologic role of heparin in the blood transport phase of fat metabolism. The concept has been strengthened by identification(3) of the tissue enzyme, lipoprotein lipase, of which heparin is probably a component(4), and demonstration (5) of a similar lipolytic enzyme in the plasma of some normal individuals. The inverse correlation found between human plasma heparin levels and serum low density lipoproteins(6) added further evidence. This report will present data showing an increase in the level of plasma anticoagulant substances (heparins) following administration

of intravenous fat emulsions in man, indicating that fat intake is a stimulus for release of heparin into the bloodstream.

In preliminary experiments(7) increase in plasma metachromatic substances was found in 6 of 10 individuals following intravenous injection of cottonseed oil emulsion. The method used for extraction and separation of endogenous circulating heparin in the earlier study was a modification of the technic suggested by Nilsson and Wenckert(8) in which heparin is adsorbed on tricalcium phosphate gel, then eluted with citrate, and the octylamine-brucine method of Jaques(9) applied to the eluate. However, the use of this method of heparin extraction was not continued since other metachromatic substances, such as chondroitin sulphate, may be present in blood. Moreover, it was found that the octylamine

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precipitation of endogenous heparin, whether protein-bound or after preliminary tryptic digestion of the protein, varied in its completeness. Thus the investigation of the effect of intravenous fat on plasma heparin had to be repeated using a more reliable method. Furthermore, a disruption of mesenteric mast cells was observed in rats following intraperitoneal injection of fat emulsions containing phosphatide stabilizers(10). This raised the possibility, albeit improbable, that the increase observed in circulating heparin values in man after administration of intravenous fat emulsions containing soybean phosphatides was the result of mast cell damage with consequent heparin release rather than a normal physiologic response to fat intake. This necessitated the use of control emulsions containing phosphatides but no fat. The control studies were additionally valuable since they were also a check upon the contingency that variations in heparin levels resulted from the emotional stress associated with the experimental procedure.

Procedure and methods. The fat emulsion used was Lipomul[†] which contains 15 g cottonseed oil, 4 g glucose, 1.2 g soybean phosphatide and 9.3 g Pluronic F 68 per 100 ml. The control emulsion was identical in all respects except for the absence of the cottonseed oil. Intravenous injections were given at a rate of 20-25 drops per minute for one hour (approximately 100 ml) and then discontinued. During administration of the fat there was a slight rise in temperature in 2 individuals, but no other reactions occurred. There was no systemic evidence of hemolysis with this small total dose of intravenous fat although slight hemolysis was noted during separation of the plasma in a few instances when the lipemia was maximal. (Hemolysis was not a possible source of error in these studies since we have found on several occasions that heparin was not present after the hemolysis of washed erythrocytes.) Hospitalized patients were used in this study. No subjects were selected who had evidence of fat metabolic or hematologic disorders, and

none had received heparin. Blood was drawn in the post-absorptive state just prior to the onset of the intravenous emulsion, 15 minutes and one hour after it was started, and approximately one hour after it was discontinued. It was placed in oxalated tubes, the plasma was immediately separated, and duplicate analysis of 5 ml aliquots was started the same day. The procedure used for extraction and assay of endogenous circulating heparin has been previously published(11). The method is based upon tryptic digestion of total serum or plasma proteins previously precipitated by methanol-acetone, dialysis after denaturation of the enzyme, and subsequent lyophilization or evaporation. The anticoagulant assay is performed as previously described(12) using recalcified sheep plasma according to the U.S.P. #14 technic. However, since the final extract after dialysis is at neutrality, no adjustment of pH is necessary. The evaporated (or lyophilized) material is dissolved in 1.0 ml of isotonic saline for the anticoagulant assay. Determination of the clotting endpoint is facilitated when evaporation rather than lyophilization has been used.

Results. The findings are presented in Table I. Fourteen individuals received both Lipomul and the control solution (Lipomul without fat), and in 4 additional subjects only Lipomul was given. The averages of duplicate analyses are shown. The maximum deviation from the mean encountered in this study was 1 unit per cent, or 11% from the mean. Accordingly changes in heparin differing less than 1 unit % from the fasting level were considered possibly due to variations in the method.

Of the 14 subjects who received both intravenous solutions, 9 showed increases in circulating heparin after Lipomul and in 4 of these heparin levels were also increased after the control injection. Twelve of the total number of 18 patients had increased plasma heparin levels after intravenous Lipomul, in one patient (L.A.) apparently a decrease occurred. The increase usually was found in the 15 minute sample, but occasionally did not appear until one hour after the intravenous was begun. In one patient the rise in heparin

[†] Generously supplied by Dr. E. A. Hawk of Upjohn Co., Kalamazoo, Mich.

TABLE I. Endogenous Plasma Heparin Values before and after Intravenous Lipomul, with and without Fat, in 18 Patients.

| Age | Sex | Time of blood sample | Heparin levels in units, % | | Age | Sex | Time of blood sample | Heparin levels in units, % | |
|-----|-----|----------------------|----------------------------|---------|-----|-----|----------------------|----------------------------|---------|
| | | | Lipomul | Control | | | | Lipomul | Control |
| 23 | ♀ | Before I-V | 12.3 | 12.5 | 44 | ♀ | Before I-V | 10 | 10.5 |
| | | I-V 15' | 14.3 | 12.7 | | | I-V 15' | 9.5 | 10.1 |
| | | " 60' | 12.7 | 11 | | | " 60' | 9.3 | 9.0 |
| | | 60' after I-V | 12.2 | 11.7 | | | 60' after I-V | 10 | 11.2 |
| 61 | ♂ | Before I-V | 10.6 | 9.6 | 26 | ♀ | Before I-V | 9.5 | 9.5 |
| | | I-V 15' | 9 | 9.6 | | | I-V 15' | 12 | 9.2 |
| | | " 60' | 9 | 9.2 | | | " 60' | 12.5 | 9 |
| | | 60' after I-V | 9 | 9.7 | | | 60' after I-V | 11.8 | 8.3 |
| 64 | ♀ | Before I-V | 10 | 8.8 | 28 | ♀ | Before I-V | 8.2 | 9.2 |
| | | I-V 15' | 9.5 | 8.2 | | | I-V 15' | 8 | 8.2 |
| | | " 60' | 10.2 | 8.2 | | | " 60' | 9.5 | 7.8 |
| | | 60' after I-V | 11 | 8.2 | | | 60' after I-V | 9 | 9.5 |
| 52 | ♂ | Before I-V | 10.2 | 10.2 | 66 | ♀ | Before I-V | 10.2 | 11.7 |
| | | I-V 15' | 12.5 | 12.2 | | | I-V 15' | 11.8 | 11.2 |
| | | " 60' | 11 | 10 | | | " 60' | 11.5 | 12 |
| | | 60' after I-V | 12.5 | 10.2 | | | 60' after I-V | 11.2 | 11.7 |
| 52 | ♀ | Before I-V | 9 | 10.5 | 61 | ♂ | Before I-V | 11.5 | 10.9 |
| | | I-V 15' | 11.5 | 12.5 | | | I-V 15' | 11.7 | 10.5 |
| | | " 60' | 9.5 | 10 | | | " 60' | 11.5 | 10.7 |
| | | 60' after I-V | 10.2 | 10.8 | | | 60' after I-V | 11.5 | 11.5 |
| 57 | ♂ | Before I-V | 11.2 | 12.2 | 43 | ♀ | Before I-V | 10.5 | 11 |
| | | I-V 15' | 11 | 10.5 | | | I-V 15' | 14 | 13 |
| | | " 60' | 10.5 | 10.2 | | | " 60' | 13.5 | 11.7 |
| | | 60' after I-V | 10.5 | 9.5 | | | 60' after I-V | 10 | 11.5 |
| 71 | ♂ | Before I-V | 11.5 | 10 | 46 | ♂ | Before I-V | 10.5 | 11.2 |
| | | I-V 15' | 13.7 | 11.7 | | | I-V 15' | 9.6 | 11.2 |
| | | " 60' | 13 | 13 | | | " 60' | 10.5 | 11.3 |
| | | 60' after I-V | 9.5 | 11 | | | 60' after I-V | 10.5 | 11.5 |
| 40 | ♀ | Before I-V | 10.8 | | 67 | ♂ | Before I-V | 9.5 | |
| | | I-V 15' | 11.5 | | | | I-V 15' | 11.5 | |
| | | " 60' | 10.3 | | | | " 60' | 13.7 | |
| | | 60' after I-V | lost | | | | 60' after I-V | 14 | |
| 78 | ♂ | Before I-V | 13.5 | | 54 | ♂ | Before I-V | 12.2 | |
| | | I-V 15' | lost | | | | I-V 15' | 15 | |
| | | " 60' | 14.5 | | | | " 60' | 12 | |
| | | 60' after I-V | 13.2 | | | | 60' after I-V | 9.5 | |

was found after the intravenous infusion was discontinued. In some individuals the increase in heparin was maintained after the fat drip was discontinued; in others it was not. In 2 cases, when a rise in heparin was found during the infusion of Lipomul, the level one hour after cessation of the intravenous was markedly below the fasting level. It is also of interest that in 3 of 4 patients in whom heparin levels rose after the control injection of Lipomul without fat, the increase was not sustained as it was in the individuals after Lipomul.

A statistical analysis of the results is shown in Table II. In the 14 subjects who

received both Lipomul and the control injection the different incidence (9:4) of increased circulating heparin is almost statistically significant. When the entire group of 18 individuals is analyzed, the results are statistically significant, the difference in proportions (12 of 18 : 4 of 14) being more than twice its standard error. Chi square analysis of the number of increased and decreased heparin levels in the 18 patients after Lipomul (12:1) shows that these results are definitely significant. It is also noteworthy that in 8 of the 12 patients in whom a rise in heparin levels after Lipomul was found, the increase was from 2-4.5 units %, or at least

TABLE II. Summary of Data and Statistical Analysis.

| | No. | >Heparin after Lipomul | >Heparin after control | Statistical method | Re- sults | Confi- dence level, % |
|---|-----|------------------------------|---|---------------------------------|--------------|-----------------------------|
| Patients who had I-V Lipomul and control (Lipomul without fat) | 14 | 9 of 14 | 4 of 14 | Chi ² test | 3.6 | Almost 5 |
| | | | | S.E. of diff. in proportions | .188 | " |
| | | Avg 21% | Avg 18% | Diff. in pro- portions | .357 | |
| Total No. of patients who received I-V Lipomul | 18 | 12 of 18 | 4 of 14 | Chi ² test | 4.6 | 5 |
| | | | | S.E. of diff. in proportions | .179 | 5 |
| | | | | Diff. in pro- portions | .381 | |
| | | Avg 22% | <Heparin after Lipomul 1 of 18 | Chi ² test | 8 | 1 |

twice the maximum deviation from the mean.

Discussion. The isolation of metachromatic substances from the blood, or evidence obtained by the use of protamine titration technics, does not prove the presence of circulating heparin. However, the demonstration of biologic (anticoagulant) activity of plasma extracts in normal humans is more convincing. This was independently accomplished several years ago by two groups(8,12) of investigators. Furthermore, electrophoresis on toluidine blue paper of the final extract obtained with the extraction method described in this study showed that it contained a metachromatic substance having the same mobility as commercial heparin(11). There are other acid mucopolysaccharides, chondroitin sulphate and beta-heparin, present in normal serum(13) and probably in the end-product we obtain. However, chondroitin sulphate has no anticoagulant activity, and beta-heparin possesses anticoagulant activity only with the thrombin method of titration. It has merely trace anticoagulant activity when tested on ox blood *in vitro*, in dogs and cats *in vivo*, or by the U.S.P. 14 method for heparin assay(14). Since the latter is the assay technic employed in this study, and the extract is dialyzed to remove false activity, it is reasonable to believe that the final titration is specific for biologically active anticoagulant heparin.

Lipoprotein changes have been demon-

strated in humans following intravenous fat emulsions(15) that are identical with those induced by the injection of heparin in normal persons during alimentary hyperlipemia. It was subsequently found that plasma lipemia clearing and lipolysis was increased in some individuals after intravenous(16,17) and oral fat intake(18). The enhanced activity, when present, was identified by inhibitor studies as lipoprotein lipase(17,18). The results reported in this paper of an average increase of 22% in circulating heparin after intravenous fat in 12 of 18 individuals conform with and extend these previous observations.

It was observed in this study that changes in the level of lipemia clearing activity do not necessarily parallel the fluctuations in circulating heparin. Since heparin is apparently a component of lipoprotein lipase(4), this observation appears paradoxical. Evidence has been presented, however(17,18), which suggests that lipemia clearing activity is inactivated during the clearing process. The quantity of circulating clearing factor demonstrable at any given moment is then a resultant of its stimulation and production on the one hand, and its inactivation by the liver (19) and the clearing process itself on the other. This may account for the fact that endogenous plasma lipoprotein lipase is not found in many individuals.

The site(s) at which lipolysis of neutral-fat containing lipoproteins occurs *in vivo* is

not clear. Lipoprotein lipase activity has been demonstrated in tissue(3) and in the bloodstream(5,16). The serum enzyme activity as measured *in vitro* accounted for the clearing of only 5% of the fat actually removed from the blood(16), and the authors therefore postulated that tissue clearing factor was primarily responsible. However, circulating lipoprotein lipase activity is usually more abundant in plasma rather than serum(20), may be inactivated by the process of lipolysis as discussed above, and the maximum demonstrable in a test tube *in vitro* where the end products of the reaction are not removed is almost certainly the minimum which occurs in the bloodstream. Moreover, in several individuals as much as 5-6 milliequivalents per liter per hour of unesterified fatty acids was released upon incubation of lipemic plasma at 37°C *in vitro*(21). The fairly rapid release of additional heparin into the blood after intravenous fat suggests but does not prove that the lipolysis of chylomicra occurs predominantly in the plasma.

Since an increase in heparin levels was found in 12 of 18 subjects after Lipomul, and in only 4 of 14 after the control injection which also contained the soybean phosphatide, the latter appears to have been eliminated as the major cause of the rise in circulating heparin through its toxic effect on mast cells (10). Furthermore, emotional factors cannot be implicated. It seems reasonable that the results obtained were primarily a consequence of the rapid entrance of fat into the blood.

Summary. 1. Plasma heparin levels were determined in duplicate in 18 individuals before, during and after intravenous infusion of cottonseed oil emulsion, and in 14 of the group after control injection without fat. 2.

There was an average increase of 22% in circulating heparin in 12 of the 18 subjects after the fat emulsion; an average increase of 18% in 4 of 14 patients after non-fat emulsion. 3. The results indicate that fat intake is a stimulus for the release of heparin into the bloodstream.

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Effect of Growth Hormone on Acetic Acid-Extractable Collagen of Hamster Skin. (23724)

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Unpublished work of Dr. John Berg* indicated that growth hormone given to old hamsters increased the acetic acid-soluble collagen in the skin. The present experiments were designed to test the validity of this observation.

Preliminary data. At the outset it was necessary to determine changes with age in the concentration of acetic acid-soluble collagen in skin of untreated hamsters. A simple procedure for doing this has been reported (1). In the present experiments the hair was removed with clippers and the skin was scraped free of subcutaneous fat. One tenth g of skin, retained in a single piece as far as possible, was placed in 5 cc of dilute acetic acid in a covered stender dish at refrigerator temperature for 24 hours. About 1.5 cc of the extract was then placed in a 75x10 mm test tube and approximately 0.3 g of salt added. The amount of collagen precipitate which resulted was graded 0 to 4 plus as a rough quantitative measure of the collagen extracted. A few strands of precipitate floating to the top was 1 plus; a veil forming over the salt and floating to the top, a 2 plus and a dense precipitate sticking to the salt, a 3 plus. A 4 plus indicated the collagen solution was so concentrated that the salt was immediately surrounded by a jel which caused the salt granules to stick together forming a mass at the bottom of the test tube. In many instances the precipitate was also measured after diluting the original extract 1:8. This dilution was made by adding to 0.25 cc of the extract 1.75 cc of dilute acetic acid. Extracts were made using both 1:1000 and 1:3000 acetic acid in distilled water. The difference in acid concentration used for extraction did not seem to affect the type but only the quantity of the precipitate obtained. Since the abdominal skin contains a higher concentration of acetic acid-soluble collagen than the

skin of the back, both were tested. The results of these tests on untreated male golden hamsters from the National Institutes of Health Colony showed the concentration of acid-soluble collagen in the skin to be less in the older animals than in the younger. Using 33-day-old animals the extracts of back skin gave 4 plus precipitates even when extracted with 1:3000 acetic acid. By 64 weeks the collagen extract of the back skin using 1:1000 acetic acid was 3 plus and using a 1:3000 acetic acid dilution for extraction it was 2 plus. By 30 months the corresponding values had fallen to 2 plus or less using the 1:1000 extract and were no more than 1 plus using the 1:3000 extract. The abdominal skin seemed to have a slightly lower concentration of acetic acid-soluble collagen at 64 weeks than at younger ages but 4 plus precipitates were still present. By 29 months 1 and 2 plus precipitates were obtained from 1:1000 acetic acid extracts of abdominal skin. From these results it was felt that if a difference existed in the concentration of acid-soluble collagen in the skin of treated animals from that in control animals it could be detected starting with hamsters about 60 weeks of age.

Growth hormone. Armour lyophilized veterinary growth hormone, lot #R-377-237 was used for experiments I and II and lot #R50109[†] for Exp. III. Data from the Armour laboratories indicated the potency of lot R-377-237 was 137% of the Armour Standard, the thyroid stimulating hormone 0.11 ± 0.02 U.S.P. unit/mg, oxytocin 0.6 U.S.P. unit/50 mg, pressor effect less than 1 unit/50 mg and depressor effect 5 γ estimated per 50 mg. Lot R50109 was 106.2% of Armour Standard, thyroid stimulating hormone 0.008 U.S.P. unit/mg, vasopressin 0.01 U.S.P. unit/mg, oxytocin 0.008 U.S.P. unit/mg, prolactin 0.1 International unit/mg and almost no adreno-corticotrophic hormone, fol-

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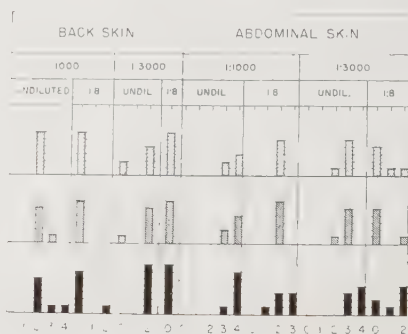
[†] Gift of the Endocrinology Study Section, N.I.H.

hicle stimulating hormone or luteinizing hormone. For Exp. I and II the growth hormone solution for injection was prepared by suspending it in 0.9% NaCl made alkaline by adding one drop of N/10 NaOH to 20 cc. The solution was kept refrigerated and only 5 cc of the hormone was prepared at one time so that a fresh solution was available approximately every 6 days. For Exp. III the NaOH was omitted.

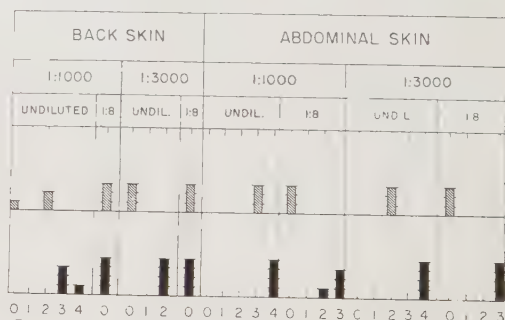
Methods. Male golden hamsters from the Nat. Inst. of Health Colony were used. The animals were housed in individual cages, given water and fed on Purina chow and carrots. The experiment was performed 3 times. In Exp. I 7 hamsters, 62 weeks of age, were injected intraperitoneally with 0.1 mg of growth hormone in 0.2 cc of alkaline saline 6 days a week for 46 days. Six uninjected controls and 6 controls injected intraperitoneally with alkaline saline were used. Exp. II consisted of 4 hamsters 83 weeks of age, injected intraperitoneally with 0.2 mg of growth hormone in 0.2 cc of alkaline saline 6 days a week for 54 days, and 3 uninjected controls of the same age maintained concurrently. Exp. III consisted of 2 hamsters 29 months and one 31 months of age given growth hormone intraperitoneally equivalent to that used in Exp. II. This treatment was continued for 51 days. Two controls 29 months of age were maintained concurrently. In all instances a complete autopsy was performed on each animal and all organs were weighed. Acetic acid-extractable collagen was determined on both back and abdominal skin as previously described; the back skin being taken at the level of but not including the sex spots and below.

Results. Exp. I. The values for the acetic acid-extractable collagen in the skin are given in Fig. 1A. There is essentially no difference between the uninjected and alkaline saline injected controls. On the average the growth hormone injected animals had more acid-soluble collagen in the skin of the abdomen than the control animals. There was very little difference between the control and experimental groups in the skin of the back. The average weight gain of the growth hormone treated animals during the experiment

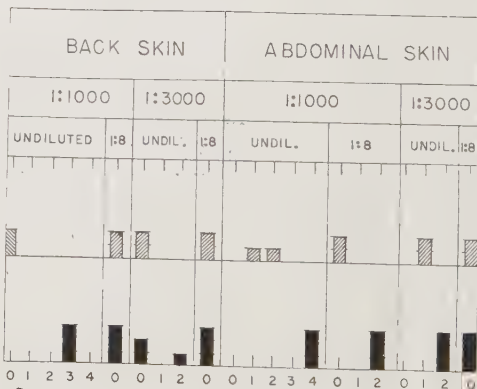
was 12 g whereas the control animals injected with alkaline saline lost 1 g. Sterile precautions were not taken with these animals and many of the injected animals had an infectious process such as peritonitis, epididymitis



A. UNINJECTED
GROWTH HORMONE INJECTED
SALINE INJECTED



B.



C.

FIG. 1. Effect of intraper. injections of growth hormone on concentration of acetic acid-soluble collagen in skin of hamsters. Each line in a column represents one animal. A. Hamsters 62 wk old. 0.1 mg growth hormone 6 days a week for 46 days. B. Hamsters 83 wk old. 0.2 mg growth hormone 6 days a week for 54 days. C. Hamsters 29 and 31 mo old. 0.2 mg growth hormone 6 days a week for 51 days.

or orchitis.

Exp. II. The values for the acetic acid-extractable collagen in the skin are given in Fig. 1B. In all instances the growth hormone treated animals had a higher concentration of acetic acid-extractable collagen in the skin of both the back and abdomen than did the controls. Experimental animal No. A4 was small with severely scarred kidneys but the amount of extractable collagen in the skin was still higher than in the controls. Using 1:1000 acetic acid, the back skin of the experimental animals gave 3 and 4 plus precipitates and the controls 0 and 2 plus. Using abdominal skin, the treated animals gave a 4 plus precipitate and the controls 3 plus. When diluted 1:8 all precipitates for back skin were 0 and those of the abdominal skin were 2 plus and 3 plus for the experimental animals and 0 for the controls. Using 1:3000 acetic acid for extraction, the differences were even more pronounced. The skin of the back from the experimental animals gave a 2 plus reaction and the controls 0. The abdominal skin of the experimental animals was 4 plus and the controls 2 plus. When the 1:3000 acetic acid extract was diluted 1:8, the back skin of both experimental and control animals was 0, while the abdominal skin of the experimental animals was 3 plus and the controls again were 0. The growth hormone treated animals gained an average of 22 g and the controls 9 g. None of the animals had peritonitis, epididymitis or orchitis.

Exp. III. The values for the acetic acid-soluble collagen in the skin are given in Fig. 1C. For both the abdominal and the back skin there was an increase in the concentration of acetic acid-soluble collagen of the growth hormone injected animals as compared to the controls. There were no infections as the result of the injections in these animals.

In both Exp. I and Exp. II there was no pronounced difference in the weight of organs or their histologic appearance that could be attributed to the effect of the growth hormone. The possible changes as the result of age preclude a statement as to the effect of growth hormone on the very old animals in Exp. III. The number of animals was small

and discouraged statistical analysis.

Discussion. In Exp. I there was an indication that the growth hormone increased the amount of soluble collagen in the skin of the old hamsters. Exp. II was modified to amplify this effect if such existed. That is to say, older hamsters were used and a larger dosage of the growth hormone was given for a longer period of time. The results of this second experiment seem to indicate that the concentration of acid-soluble collagen in the skin of old hamsters is increased by the administration of growth hormone. In Exp. III still older hamsters were used and here the results confirmed the effect of growth hormone in increasing the acetic acid-soluble collagen component of both back and abdominal skin. The growth hormone appears to alter the collagen metabolism rather than to maintain it at the younger level. The older animals in Exp. III, Fig. 1C, which were treated with growth hormone have a higher concentration of acetic acid-soluble collagen in the skin than do the younger untreated control animals in Exp. II, Fig. 1B. If the collagen metabolism had been maintained at a younger level rather than altered, these two groups would be more nearly alike.

In the present study the animals were mature, even old, yet the alteration of collagen metabolism still occurred on the administration of growth hormone. They did increase in weight but whether this reflects an anabolism of structural proteins including collagen is not known. On gross observation of skin strips taken from over the right thigh no definite change in the thickness could be detected from that of the controls. Whether the old collagen is changed, new collagen is being laid down, or both, requires further investigation. The gross and microscopic observations of the tissues in these animals were not useful in deciding whether the growth hormone was acting through one or more intermediate endocrine glands.

Summary. Concentration of acetic acid-extractable collagen in the skin of hamsters was used as a measure of the effect of growth hormone on collagen metabolism. The intraperitoneal administration of growth hormone increased the concentration of the acetic

acid-soluble collagen in the skin of old hamsters.

technical assistance.

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Antigenic Relationships in Insect Extracts.* (23725)

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Recent findings have indicated that dust from disintegrated insects may constitute a common type of antigen responsible for asthma and hay fever(1). One of the problems raised in this connection is whether the observed group reactivity to insects in allergic subjects is due to identical allergens in the extracts of various insects. An unequivocal answer may be obtained only by isolation and comparison of purified allergens from different insects. Useful information about the relationship of extract constituents which may also serve as a guide to fractionation of insect extracts may, however, be obtained by immunological study.

Antigens of several grass pollen extracts were studied by Gosselin *et al.*(2) using hemagglutination. They found many cross reactions among the grasses but also a high degree of specificity by inhibition technics. Gel diffusion has been applied extensively to allergen extracts by Wodehouse(3). Although our plan of study of antigenic relationships of insect extracts included such technics as anaphylactic shock, the production of asthma in the guinea pig from antigen aerosols, Schultz-Dale reaction and passive transfer reactions with reagins, the present study is confined to hemagglutination and specific precipitation in agar.

Materials and methods. The insects used for this study were chosen to satisfy the following requisites: (1) previously demon-

strated whealing skin reactions and clinical allergy in man; (2) close taxonomic relationship between some forms; (3) fairly distant biologic relationship between others; (4) availability of adequate quantities of a pure breed of insect. *Insects.* Mayfly (*Ephemoptera*), fly (*Phormia regina*), cricket (*Acheta domestica*), cockroach (*Blatella germanicum*) and silk pupa (*Bombyx mori*) were dried, ground, the ether soluble fraction discarded and dried again. *Extracts.* One gram of dried powder was extracted overnight in 10 ml saline with shaking. Extracts were clarified by centrifugation, merthiolate was added and the solution was frozen. This extract was designated 1:10, and subsequent dilutions were expressed on this basis. *Antisera.* Rabbits were immunized with 4 ml of 1:10 extract in Freund's adjuvant. After 4 weeks test bleedings were made and the gel precipitin pattern observed. Where necessary a second injection was made at this time. Sera were prepared from periodic bleedings and pools made in accordance with gel precipitation data. *Hemagglutination.* The tannic acid technic of Boyden(4) as modified by Stavitsky(5) was employed both for hemagglutination and hemagglutination inhibition titers. Hemagglutination inhibition was performed by preincubating 4 agglutinating doses of serum in all tubes with graded dilutions of antigen followed by addition of coated cells. Extracts were usually diluted to 1:100 for the purpose of coating sheep cells since more concentrated extracts often caused spontaneous agglutination or lysis. *Agar Diffusion.* The triangle plate method of Jennings

* This research was supported by research grants from Nat. Inst. of Allergy and Infectious Diseases, and Asthmatic Children's Aid, Chicago, Ill.

TABLE I. Hemagglutination Titers of Antisera to Insect Extracts.

| Antigen* | Sera | | | | |
|----------|--------|-------|-------|---------|-------|
| | Mayfly | Fly | Roach | Cricket | Silk |
| Mayfly | 1600 | 6400 | 0 | 800 | 0 |
| Fly | 0 | 10240 | 1600 | 800 | 20 |
| Roach | 6400 | 0 | 10240 | 800 | 0 |
| Cricket | 0 | 25600 | 800 | 10240 | 20 |
| Silk | 0 | 0 | 0 | 20 | 25600 |

* Tannic acid treated sheep cells coated with insect extract.

and Malone(6) was used. Plates were placed in moistened Petri dishes and covered with another dish of the same size and sealed with scotch tape to prevent desiccation. Patterns were generally recorded after development for 1 week at room temperature.

Results. Hemagglutination. Extensive cross reactions shown in Table I were obtained when the agglutination titer of each serum was determined against sheep cells coated with each extract. Inhibition of agglutination in the 5 homologous systems of extract coated cells and corresponding serum was appreciable only with the homologous extract as indicated in Table II. Inhibition of the above cross reactions (Table I) was often exhibited by extracts other than the cross reacting pair as is shown in Table III.

Agar precipitation. When an extract was placed in one depot of a triangle plate and its homologous serum in another, a number of bands were seen emanating as straight lines from the apex of the clear agar triangle formed between the two wells. The number of zones of precipitation obtained were as follows: Mayfly 3, fly 2, roach 6, cricket 4 and silk 3. When the third unused depot of the above experiment was filled with a heterologous extract, the pattern was virtually the

same as before. In most cases, however, an additional diffuse and faint zone was seen emanating from the adjacent apex and forming a continuous arch into the apex from which the homologous zones originated. This band indicated a cross reaction between the heterologous extract and the serum and that this antigen was similar to or identical with some component in the homologous extract.

When each of 2 troughs was filled with a different extract and the third with a serum heterologous to both extracts one diffuse band of identity was usually observed and in some cases a second similar zone appeared. Without exception all heterologous combinations resulted in at least one band of identity between both extracts. This band of identity is evidence for a common antigen in all of the extracts studied. Substitution of normal rab-

TABLE III. Heterologous Inhibition Titers of Insect Extracts.

| System | | Extracts | | | | |
|---------|--------------|----------|------|-------|---------|------|
| | | May-fly | Fly | Roach | Cricket | Silk |
| Mayfly | Anti-fly | 800 | 800 | 200 | 1600 | 0 |
| " | Anti-cricket | 1600 | 200 | 200 | 800 | 0 |
| Fly | " | 3200 | 6400 | 1600 | 6400 | 0 |
| " | Anti-roach | 0 | 6400 | 1600 | 0 | 0 |
| Roach | Anti-mayfly | 1600 | 200 | 6400 | 200 | 0 |
| " | Anti-fly | 0 | 800 | 3200 | 1600 | 0 |
| Cricket | " | 0 | 6400 | 200 | 6400 | 0 |
| " | Anti-roach | 0 | 0 | 800 | 400 | 0 |

bit serum for the specific heterologous serum above also resulted in the appearance of zones of identity. Normal serum did not reproduce any of the specific homologous zones but only the heterologous bands of identity. Normal serum of 2-week-old rabbits gave parallel results to serum from older animals. If the observed reaction with non-immune serum is due to antibody it is probably due to natural rather than acquired antibody.

Discussion. The results obtained with hemagglutination inhibition indicate that the antigens of major quantitative significance are specific for each extract since only the homologous extract could effectively inhibit a homologous reaction. The cross reactions obtained between extract coated cells and heterologous serum show that at least some

TABLE II. Homologous Inhibition Titers of Insect Extracts.

| Homologous reaction* | Extracts | | | | |
|----------------------|----------|------|-------|---------|-------|
| | Mayfly | Fly | Roach | Cricket | Silk |
| Mayfly | 25600 | 400 | 0 | 0 | 0 |
| Fly | 0 | 3200 | 0 | 0 | 0 |
| Roach | 0 | 0 | 4000 | 0 | 0 |
| Cricket | 0 | 0 | 0 | 1600 | 0 |
| Silk | 0 | 400 | 200 | 200 | 25600 |

* Reaction between extract coated cells and homologous antiserum, in fixed agglutinating concentration, in presence of dilutions of extract.

of the extracts contain similar or identical antigens in addition to the major specific antigens. Each cross reaction might represent a unique antigen common to the two interacting insect extracts or there might be an antigen common to all of the extracts. For each cross reacting pair one expects inhibition by extracts of each member of the pair involved. When extracts other than those of the cross reacting pair also inhibit this is evidence for a common antigen in all the inhibitory extracts. By this criterion there appears to be an antigen common to mayfly, fly, roach and cricket; one common to fly, roach and cricket; one common to fly and roach, and another to cricket and roach. It cannot be determined from these data whether these are single or multiple antigens. There is evidence then for cross-reacting antigens of wide and of limited distribution among the insects studied which cannot be correlated with insect taxonomy. The original source of these antigens is probably the insects themselves but could also be due to fortuitous factors such as common bacterial contaminants.

Gel diffusion studies were initiated in part to confirm and amplify the findings in hemagglutination. Although apparent confirmation of extensive cross reactions was obtained, normal rabbit serum produced the same patterns as anti-insect rabbit serum. Gel diffusion, therefore, can neither confirm nor negate the results obtained by hemagglutination.

This study introduces at least two interesting possibilities. One is that the observed

bands of identity with normal serum are artefacts and therefore due warning should be given those using the reported methods. The second possibility is that the bands are specific reactions between heterogenetic components of the insect extracts with some natural antibody present in rabbit serum. One type of natural antibody that might be involved is the isoagglutinin. If this is the responsible agent then one would have to conclude that the extracts contained materials related to mammalian blood group substances. Investigation of certain aspects of this problem is being made.

Summary. 1. Extracts of mayfly, fly, cockroach, cricket and silk have been investigated by hemagglutination and agar precipitation technics. 2. Evidence has been obtained for the presence of specific antigens in each extract as well as antigens common to one or more of the other extracts. 3. The minimum number of antigens in each extract has been determined by agar diffusion. 4. The agar diffusion and hemagglutination data provide information helpful in purification studies.

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Cartesian Diver Technics for Embryonic Chick Hearts.* (23726)

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The embryonic chick heart is ideal for certain metabolic studies because it is readily available, inexpensive, small enough to permit ready diffusion of dissolved gases to and from

all parts, yet large enough to be handled easily.

Such hearts, however, are too small for successful use in the conventional Warburg apparatus and too large for use in the cartesian diver as previously described (1,2,3). But, by adopting a feature of the Warburg, namely

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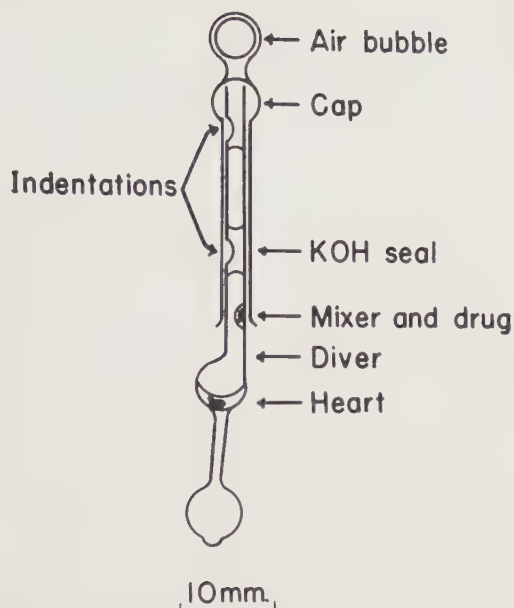


FIG. 1. A cartesian diver suitable for work with the embryonic chick heart.

shaking, to the cartesian diver, and by perfecting convenient methods for reducing loss of gas from the diver, accurate measurement of oxygen consumption by the embryonic chick heart becomes possible. In addition, by perfecting a method for mixing a drug with the solution containing the heart during the course of an experiment, observations before and after addition of the drug become practical.

Methods. Hearts from chicken eggs incubated 5 days were used; at this age the hearts

are of maximum size permitting excellent diffusion of gas to all parts, are not dependent upon a coronary circulation, and have a dry weight of about 200 μg . The hearts were removed from the embryos and the blood vessels cut from them with glass tubing pulled to a very fine point. The hearts were introduced into the mouth of the diver by simple hand-held pipets and were shaken into the bulb of the diver. All excess fluid was removed by suction, and then a measured quantity, usually 10.0 mm^3 , of Ringer or Locke solution was added. In general, the cartesian diver technics previously described(1,2,3) were employed. The temperature of the bath was $37.25 \pm 0.002^\circ\text{C}$. This limited variation in temperature was attained by operating the heating current at a voltage only a trifle more (when "on") and a trifle less (when "off") than that required to maintain the temperature of the bath. The suspension fluid was that of Holter(3). The manometer was filled with Brodie's solution(4) adjusted to a P_o of 10133. Changes in the manometric pressure required to suspend the diver stationarily were corrected for changes in atmospheric pressure read from a sensitive anaeroid barometer (Wallace and Tiernan, Belleville, N. J., Model FA139). The diver and heart were viewed through a microscope at a magnification of 40x. Properties of a diver suitable for work with the embryonic chick heart are given in Table I and Fig. 1. The indentations in the neck (Fig. 1) aid in

TABLE I. Properties of a Cartesian Diver Suitable for Embryonic Chick Heart.

| | Wt in air, mg | Wt in suspension fluid, mg* | Specific gravity | Vol, mm^3 |
|---|------------------|-----------------------------------|---------------------|---|
| Cap | 285.2 | 4.8 | 1.353 | 210.8 |
| Mixer | 5.4 | 2.2 | 2.245 | 2.4 |
| Fluids: Ringer, etc. | 10.1 | | 1.005 | 10.0 |
| Drug droplet | 1.0 | | 1.005 | 1.0 |
| 5% KOH | 10.4 | | 1.038 | 10.0 |
| Total fluids | 21.5 | | | 21.0 |
| Heart (estimated) | 1.3 | | 1.06 | 1.2 |
| Diver (No. 136) | 420.6 | | 2.23 | 188.6 |
| Vol of gas in diver when suspended stationarily | | | | 127.7 mm^3 |
| Diver constant (when P_o is 10133 and temp. 37.3°C) | | | | 1 mm = $1.11 \times 10^{-2} \text{ mm}^3$ |

* By method of Zeuthen(9).

placing the KOH seal and in filling the upper portion of the neck with suspension fluid so that the initial suspension can be accomplished at approximately atmospheric pressure. The tail of the diver is bent to allow it to lie flat against the wall of the diver holder during mixing (see below). To hold the seals and the droplet of drug (see below) in place in the neck of the diver, the interior was coated with silicone (DC-200, Dow Corning Corp., Midland, Mich., 5% in chloroform), and the divers were baked at 360°C for one hour(5,6,7). In addition the necks were dried with cotton swabs and with a small hot wire after introduction of the heart. To facilitate agitation of the fluids during shaking and to ensure mixing of the entire droplet of drug (see below), the silicone was then etched from the bulb of the diver and from the lowest millimeter of the neck of the diver with 30% sodium hydroxide solution. The diver constant was calculated as previously described(1,2,3) taking into consideration the weight and volume of the diver, of the cap, of the mixer, of the fluids, and of the heart, the latter perforce by estimation (Table I). At the close of each experiment each heart was weighed on a quartz fiber balance somewhat like that of Lowry(8). The balance had a capacity of 1200 μ g and a sensitivity of 1.5 μ g. Oxygen consumption could then be expressed as cubic millimeters per milligram of dry heart.

Caps. The wide neck of the divers and the temperature at which the experiments were conducted introduced the hazard of excessive loss of gas from the diver. Plugs and various types of seals were tried and found inconvenient to use. Caps (Fig. 1) were found easy to use, aided in keeping the diver erect, and reduced the inadvertent loss of gas virtually to zero. The caps were constructed from thin walled glass tubing selected to fit snugly yet move freely over the neck of the diver. A bubble of air was sealed into the top of the cap giving it a very high center of buoyancy. The weight of the cap was adjusted carefully by fusing in or removing glass so that the cap would sink very slowly when filled with and immersed in suspension fluid. Each cap was weighed in air and then weighed

in suspension fluid by the method of Zeuthen (9). Knowing these weights and the specific gravity of the suspension fluid, calculation of the volume of the cap is possible.

Shaking. It was soon found that oxygen did not dissolve into the fluids fast enough to meet the needs of the heart in the diver. This was remedied by shaking the divers á la Warburg. To this end the diver holder was mounted on a movable frame bearing a small motor with an eccentric weight on its shaft. The speed of the motor was controlled and kept constant with a rheostat and a constant voltage transformer. The divers were shaken at about 15 oscillations per second through an excursion of about 3 mm. Shaking with greater violence occasionally broke the seals in the diver.

Experiments next established that as the intensity of shaking was increased from least up to that usually used (attained at 80 volts) there was an increase in oxygen uptake; when the intensity was increased still more, there was no further increase in oxygen uptake. This indicates that the heart can be supplied with all the oxygen it can consume. In other exploratory experiments it was established that the oxygen uptake of hearts from embryos incubated 6 days was very appreciably more than that of hearts from embryos incubated 5 days. Since the experiments were done with 5-day hearts, it is apparent that much more oxygen was available than usually required. Vertical currents in the suspension fluid would introduce errors in the apparent pressure required to suspend a diver stationarily. Creation of such vertical currents by shaking was prevented by filling the diver holder and a few centimeters of its stem with suspension fluid. Shaking was stopped during observation on the rate and beat of the heart and during the final critical immobilization of the diver during each pressure measurement.

Mixing. In almost all experiments it was desirable to measure oxygen consumption and observe the rate and beat of the heart before and after a drug; to this end a method was sought in which a drug could be mixed with the fluid surrounding the heart followed by immediate resumption of accurate measure-

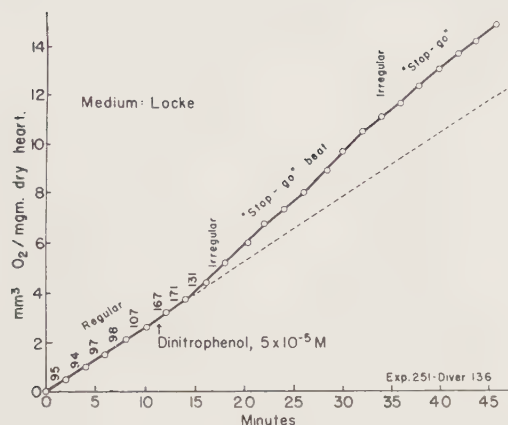


FIG. 2. Oxygen consumption and heart rate and beat before and after dinitrophenol.

ment and observation. Methods employing pressures for mixing(6,7,10,11) were found to be unsatisfactory for experiments with chick hearts: the altered pressures produced an apparent uptake or release of gas, probably by altering the quantity of gas dissolved in the fluids. The following method was found to be satisfactory. A droplet, usually 1.0 mm³, of fluid containing the drug was deposited within the neck of the diver (Fig. 1), and a glass coated iron "mixer" was pulled into it with the aid of a small magnet. After the control period, the mixer was maneuvered from outside the diver holder by means of a powerful, hand-held, permanent magnet so that the mixer was pulled slowly into the bulb of the diver, towing the droplet containing the drug with it. In this fashion mixing could be accomplished without disturbing the contents of the diver in any undesirable way. In nearly all experiments all of the droplet was mixed successfully, but in case of failure, it was practical to estimate the portion of the droplet that had been actually mixed. The construction of the mixer was quite similar to Claff's "magnetic flea"(12).

Results. The oxygen consumption of an embryonic chick heart before and after a representative drug is shown in Fig. 2. Above the curve is also shown the heart rate and certain characteristics of the heart beat. The figure shows that when dinitrophenol was added to the Locke solution bathing the heart the oxygen consumption increased appreciably

and the heart assumed a characteristic type of arrhythmia. This increase in oxygen consumption induced by dinitrophenol is comparable to that described by Lee(13).

In other experiments sodium iodoacetate was added to Locke solution bathing the heart; the oxygen consumption decreased, and the heart eventually stopped beating entirely. Such a decrease in oxygen consumption induced by iodoacetate is also comparable to that described by Lee(13).

In control experiments Locke solution was mixed with Locke solution bathing the heart; this treatment did not alter the rate of oxygen consumption nor the heart rate and beat.

Results of experiments with ouabain have been presented previously(14).

Discussion. Actual experiments indicate that there is no change in oxygen consumption when no change might reasonably be expected (control), there is an increased consumption when an increase might be expected (dinitrophenol), and that there is a decreased consumption when a decrease might be expected (iodoacetate).

Fig. 2 shows that the actual points of measurement of oxygen consumption lie within a narrow band and form a smooth curve; this indicates that shaking did not introduce errors in these measurements. Fig. 2 also shows that mixing did not distort measurement of oxygen consumption.

These methods should also be applicable to studies of tissues whose size is roughly intermediate between those that can be studied by Warburg technics and those that can be studied with the conventional cartesian diver. In addition, the use of oil seals to limit evaporation of fluids and to reduce loss of mobile gases, such as carbon dioxide, should make possible measurement of many phenomena other than oxygen consumption.

Summary and conclusion. Methods are described adapting the cartesian diver for experimentation with the embryonic chick heart. It is concluded that such methods indeed measure oxygen consumption of embryonic chick hearts with accuracy.

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Potentialation of Smooth Surface Caries by Sodium Dehydroacetate Varies Administered to the White Rat. (23727)

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In a previous study(1) dehydroacetic acid (DHA) markedly potentiated the severity of smooth surface caries in the rat when incorporated in a cariogenic diet at the 0.1% level. To extend this finding and to evaluate the possible extra-oral systemic effect of the compound on caries, DHA was administered by intubation and intraperitoneal injection as well as in the diet and drinking fluid.

Methods. The water soluble sodium salt DHA-S* was used throughout but the dosage levels are given in terms of DHA† to conform with the study previously reported(1). Litter-mated weanling, female, Sprague-Dawley rats individually housed in screen-bottomed cages received the cariogenic diet of McClure and Folk containing an autoclaved skim milk powder(2) for 60 days. The experimental design of the study is shown in Table I. Twenty-four litters of rats were used in Exp. A and B, and 40 litters in Exp. C. The diets were fed *ad libitum* in Exp. A and B. In Exp. C fresh diet was offered daily on a paired

feeding basis. Distilled water intake was *ad libitum*. The DHA drinking fluids in Experiment A varied between 0.25, 0.50, and 0.75 mg/ml, and the volumes were controlled in order to equilibrate fluid DHA intake with dietary DHA. The diet and weight gain were measured routinely. The solutions used for intubation and intraperitoneal injection contained 10.0 mg DHA/ml (12.4 mg DHA-S/ml) and were given in 2 divided doses daily except on week ends when half amounts were administered. The mean daily DHA intake by these extra-oral routes in Exp. B and C is shown in Table I. Since 0.10% DHA in the diet had markedly potentiated caries in 3 previous trials(1) and Exp. A, no dietary DHA control was included in Exp. B. To further substantiate the caries potentiating effect of parenterally administered DHA, the regimen of injection used in Exp. B was followed in Exp. C. Amounts of DHA varying from 0.60 to 1.0 ml/day were injected and control rats were injected with equal volumes of distilled water. In Exp. B and C, the rats were kept on the cariogenic diet for one week before intubation and injection of DHA, since weanling rats did not tolerate DHA by these routes of administration. After 60 days on experiment, the rats were sacrificed and the molar teeth were scored for smooth surface

* Obtained from Gaines Chemical Works, Carlstadt, N. J. Calculated for $C_8H_7Na \cdot H_2O$: C, 46.16; H, 4.36. Found: C, 46.20; H, 4.44.

† Molecular weights of sodium salt monohydrate and free acid are 208.1 and 168.1 respectively, so that an 0.124% solution of the former is equivalent to 0.10% solution of the free acid DHA.

TABLE I. Caries Experience* of White Rats Receiving DHA† in the Diet, in the Drinking Water, by Intubation and by Intraperitoneal Injection.

| Exp. No. | No. rats | Mode of DHA admin. | DHA intake (mg/day) | Carious rats (%) | Caries score/rat |
|----------|----------|--------------------------------------|---------------------|------------------|------------------|
| A | 23 | | .0 | 73.9 | 3.5 ± .9 |
| | 18 | 0.10% in diet | 5.3 ± .1 | 100.0 | 13.8 ± 2.2 |
| | 18 | Drinking water | 4.5 ± .3 | 88.9 | 9.3 ± 2.0 |
| B | 21 | 0.10% in diet | 6.4 ± .2 | 100.0 | 15.9 ± 1.0 |
| | 17 | Intubation | 6.2 ± .1 | 100.0 | 13.9 ± 2.3 |
| | 17 | Intraper. inj. | 6.2 ± .1 | 100.0 | 13.0 ± 1.8 |
| C | 40 | (H ₂ O by intraper. inj.) | .0 | 47.5 | 1.4 ± .3 |
| | 22 | Intraper. inj. | 6.3‡ | 86.4 | 9.9 ± 2.3 |

All values are expressed as mean ± S.E.

* Smooth surface caries on molar teeth. animals received identical amounts.

† Provided as sodium salt, DHA-S.

‡ All

caries according to McClure *et al.*(2). Occlusal surface caries was very limited or non-existent as seen by low power magnification. The chi square test as previously employed (3) was used to assess the difference in caries incidence, and Fisher's "t" test was used to measure the significance of the difference of all other comparisons. All estimates of errors of the means are expressed as the standard errors calculated according to Mantel(4).

Determination of DHA in Rat Saliva. Although DHA was administered parenterally, it was possible that appreciable excretion of DHA in the saliva could produce an intraoral effect. Hence, the content of DHA in pooled rats' saliva was measured at various intervals of time following intubation and intraperitoneal injection of 5.0 mg DHA (as DHA-S) in 0.5 ml H₂O. In addition, a pooled sample of plasma was also analyzed. To determine the DHA content in the saliva following its oral administration, diet 636 containing 0.10% DHA was offered to 12 rats and the intake recorded. At the end of 14 days, the rats' mouths were swabbed under nembutal to remove diet residuum. Adequate controls and recoveries were carried out. Salivas were collected according to Benarde *et al.*(5) with minor modifications‡ and assayed for DHA by the procedure of Woods *et al.*(6).

Results. In Exp. A, the rats receiving 0.10% DHA in the diet§ or the DHA drinking fluid showed significant increases in caries scores when compared with the controls ($p < 0.01$ and $p < 0.02$ respectively). In Exp. B, DHA given by intubation or by intraperitoneal injection appeared as effective in po-

tentiating caries as an equivalent amount of dietary DHA. Although the experimental regimen in Exp. C results in a low caries score as previously observed(7), nevertheless both the caries incidence and severity were again significantly increased ($p < 0.01$) when DHA was injected.

The diet intakes for the experimental rats of Exp. A and B were significantly lower than those for the controls (6.5 ± 0.2 g *vs.* 7.8 ± 0.2 g and $p < 0.01$). The weight gains were also similarly significantly reduced (1.3 ± 0.04 g *vs.* 0.9 ± 0.08 g and $p < 0.01$).

Concentration of DHA in rat saliva. The concentration of DHA in rat saliva was 3.5, 4.1, 5.5, 5.2 and 4.6 mg % at 1, 2, 3.5, 4 and 5 hours after injection of 5 mg DHA. Saliva collected from another group of rats 3.5 hours after injection of DHA contained 5.2 mg % DHA, and the blood plasma contained 11.1 mg % DHA. Rats intubated with 5 mg DHA had 5.6 mg % DHA in the saliva after

‡ The following solutions were administered by intraperitoneal injection:

1. DHA-S Solution: 12.4 mg DHA-S/ml; equivalent to 10 mg DHA/ml.
2. Nembutal solution: Abbott, Veterinary Grade 60 mg/ml. Used 1 ml of a 1:10 dilution.
3. Picrotoxin solution; K and K Laboratories, Long Island City, N. Y. Used 0.5 ml of solution containing 1 mg/ml to counteract depressant effect of nembutal on respiratory center.
4. Pilocarpine nitrate—Gane and Ingram, N. Y. City. Used 1 ml of solution containing 1 mg/ml.

§ No significant change in either incidence or severity (caries score) of caries was observed in an additional group of rats receiving 0.05% DHA in diet.

4 hours which was similar to that found after injection (5.2 mg %) of the same amount. Twelve rats receiving diet 636 containing 0.10% DHA for 14 days had a mean daily DHA intake of 10.9 ± 0.4 of DHA. Three pooled samples of saliva collected from these animals contained 15.0, 16.4 and 17.6 mg % DHA. No absorption at 310 $m\mu$ (wave length at maximum density) was found in the control samples, and 95% of the DHA added to rat saliva and blood could be recovered.

Discussion. The present findings corroborate previous studies(1) that 0.10% DHA in diet 636 has a pronounced potentiating effect on the incidence and severity of smooth surface caries. These additional studies have indicated that DHA also has a marked caries potentiating effect when administered by intubation or by intraperitoneal injection.

Of primary interest is the possibility that an extra-oral systemic effect of DHA may be involved in its caries potentiation. Although administration of DHA by intubation and injection precludes any direct contact of the compound with the oral cavity, it is apparent that its secretion in the saliva is an important consideration. Rather extensive studies demonstrated that appreciable concentrations of DHA in rat's saliva resulted from dietary as well as intubated and injected DHA. Hence, it appears that the caries potentiating effect of DHA by these routes may be indirectly mediated through its secretion in the saliva, even though the rate of flow would be presumed to be less under normal physiological conditions than under pilocarpine stimulation.||

Our results, nonetheless, do not eliminate the possibility that systemic factors may be

operative in the potentiation of caries. The idea that systemic factors may be concerned in experimental dental caries has received further attention in this laboratory. Thus with a lysine deficient cariogenic diet, intubated lysine has been shown to be cariostatic although no significant elevation of lysine was noted in the rats' saliva(8). In addition, intubated EDTA also potentiated caries. However, its concentration in saliva was not determined(9).

Summary. 1. The ingestion of DHA in the diet, drinking water, by intubation and by intraperitoneal injection all had a pronounced potentiating effect on the smooth surface caries produced in white rats by a heat processed skim milk powder diet. 2. Considerable concentrations of DHA were found in stimulated rat saliva following its administration by diet, by intubation and by intraperitoneal injection. 3. It appears that the potentiating effect of DHA on smooth surface rat caries cannot be attributed unequivocally at this time to an extra-oral systemic effect.

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|| Under pilocarpine stimulation approximately 1 ml of saliva was collected in 20 minutes, after which little saliva was secreted.

Iodine-131 Labeled Cholografin Studies in Dogs with Normal and Abnormal Liver Physiology. (23728)

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(Introduced by Barnes Woodhall)

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Langecker(3) first described a new chemical compound 2,4,6-Tri Iodo-3-acetylamino Benzoic acid. In the same year Frommhold (1) and Hornykiewytch(2) demonstrated the value of the sodium salt of this new compound (cholografin) as an intravenous cholangiographic agent. Frommhold in his early paper suggested that this compound might be useful as a liver function test. More recent work(4) has shown a correlation between failure of opacification of the biliary tree after the intravenous injection of cholografin and liver diseases. Inasmuch as cholografin is readily tagged with I^{131} and the excretion of such a compound could be easily followed, it was thought that cholografin tagged with I^{131} warranted a study to determine if it might be used as a liver function test. This study reports the build-up of radioactivity over various organs, clearance rate of the radioactivity from the blood, and excretion of radioactivity in the urine following intravenous injection of cholografin tagged with I^{131} in normal dogs and in dogs in which liver physiology had been altered.

Method. Four normal dogs, a dog in which the common bile duct had been ligated, and a dog in which a portal-caval shunt had been performed were studied after being fasted and dehydrated for 12 hours. Following the injection of 12 microcuries of cholografin* labeled with I^{131} , the radioactivity was determined anteriorly over the heart, liver, and bladder using a scintillation detector with a count rate meter. The clearance rate from the blood was determined by counting blood samples drawn at intervals in a well scintillation counter. When possible the dogs were catheterized and the radioactivity of the urine determined. The tagged cholo-

grafin was diluted with untagged sodium cholografin† to a total dose of one half ml/kg of body weight. A dog on which normal values had been determined was explored and the common bile duct was ligated. Subsequent studies with tagged cholografin were repeated. A second dog was anesthetized a number of times with carbon tetrachloride. After each anesthesia, the study was repeated. Thyroid uptake studies were done on a number of dogs to determine if any free iodine had been concentrated by this organ.

Results. On the 4 normal dogs it was found that the approximate time to clear one-half the radioactivity from the blood stream averaged 74 minutes with a range of 71 to 80 minutes. The time required to clear the blood of one-half the radioactivity in dogs in which the common duct had been ligated was significantly increased to 108 minutes or greater as shown in Fig. 1 and 2. No significant change in the clearance rate was observed whether the dogs were hydrated or dehydrated.

Fig. 3 shows the change in clearance rate of radioactivity from the blood produced by carbon tetrachloride anesthesia. The time necessary to clear one-half the radioactivity from the blood was increased from 71 minutes before administering carbon tetrachloride anesthesia to a value in excess of 120 minutes after the second and third anesthesia.

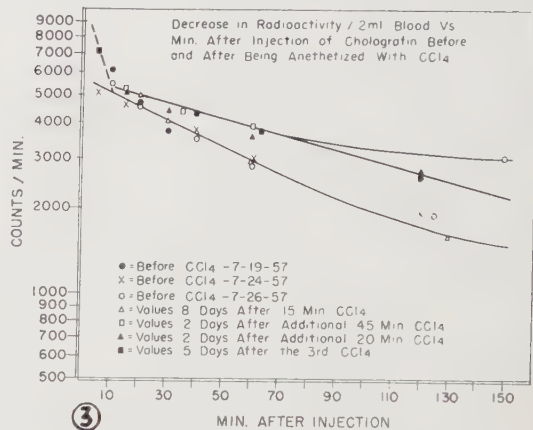
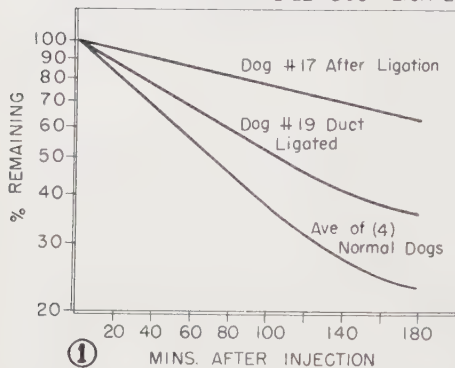
Following a portal-caval shunt the clearance of radioactivity from the blood was similarly delayed as shown in Fig. 4.

When the radioactivity was monitored over various organs no differences were noted whether the dogs were hydrated or dehydrated except for possibly a slightly higher radioactivity over the bladder in the hydrated dogs. Unfortunately sufficient urine samples could not be obtained to substantiate this.

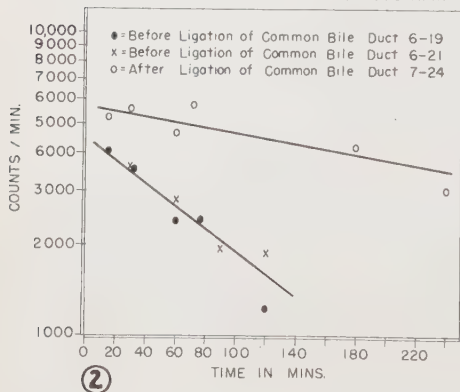
* Generously supplied by Dr. Paul Numerof, of the Radioisotope Department, Squibb Institute, New Brunswick, N. J.

† Generously supplied by Mark H. Lund, of Squibb Institute, New Brunswick, N. J.

CURVES SHOWING THE DIFFERENCE IN THE CLEARANCE OF RADIOACTIVITY FROM THE BLOOD OF NORMAL DOGS & THOSE WITH THE COMMON BILE DUCT LIGATED



DECREASE IN RADIOACTIVITY / 2ML. BLOOD VS MINS AFTER INJECTION OF CHOLOGRAFINS



CLEARANCE OF RADIOACTIVITY FROM BLOOD AFTER A PORTAL-CAVAL SHUNT

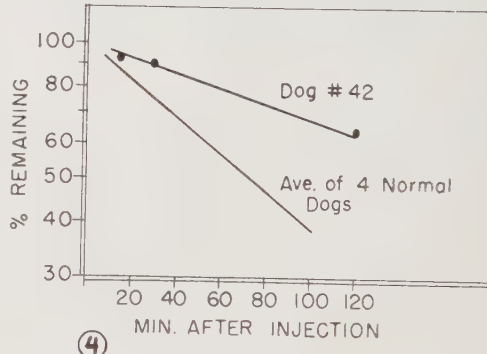


FIG. 1-4.

Representative curves of radioactivity over the liver and heart of normal dogs and of dogs with disturbed liver function are shown in Fig. 5. In all the normal dogs there was

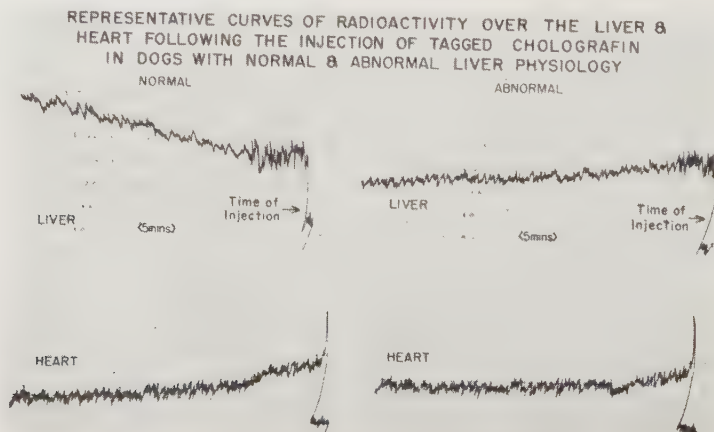


FIG. 5.

TABLE I. Percent of Total Radioactivity Excreted in Urine for Time Intervals Shown before and after CCl₄ Anesthetics.

| No. of CCl ₄ anesthetics | % excreted |
|-------------------------------------|----------------|
| Before any CCl ₄ | 1.5 in 60 min. |
| 8 days after 1st | 3.1 60 " |
| 2 " " 2nd | 5.0 35 " |
| 2 " " 3rd | 6.1 30 " |
| 5 " " 3rd | 5.2 30 " |

concentration of at least 20% radioactivity over the liver, whereas in dogs with disturbed liver physiology there was no concentration or an actual decrease of radioactivity over the liver. The decrease of radioactivity over the heart was similar to the clearance of radioactivity from the blood and in general the curve fell more rapidly in the normal dogs. Generally the radioactivity over the bladder rose to higher levels in the dogs with disturbed liver physiology.

No concentration of radioactivity could be detected in the thyroid glands of the dogs.

The percent of radioactivity excreted in the urine was calculated after each of the carbon tetrachloride anesthetics. A progressive increase in the amount of radioactivity excreted was found (Table I). A routine urinalysis performed 5 days after the third carbon tetrachloride anesthesia was normal.

The dog on which a portal-caval shunt had been performed showed a high urinary excretion of 11.5% in 60 minutes.

In Fig. 3 the value for clearance of radioactivity from the blood 8 days after administration of 15 minutes of anesthesia with carbon tetrachloride is the same as the normal curve. This is explained by the small amount of anesthesia given and the 8-day de-

lay which is sufficient for liver regeneration.

Frommhold in his early work stated that the iodine was very tightly bound to cholografin. The lack of radioactivity over the thyroid indicates that insignificant free iodine is present in the tagged cholografin at the time of injection or is liberated from the cholografin by the liver or other organs.

It has been shown with tagged rose bengal (5) that the ligation of the common bile duct of dogs did not alter concentration in the liver but increased concentration of radioactivity in the gall bladder. In this study, concentration of radioactivity, as determined over the anterior aspect of the liver, decreased following duct ligation.

Conclusion. 1) After injection of cholografin tagged with I¹³¹ in normal dogs and in dogs with altered liver function, there are sufficient differences in concentration of radioactivity in the liver to warrant further investigation of this material as a possible liver function test in human patients. Significant differences are also noted in the clearance rate of radioactivity from the blood and excretion in the urine.

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Sulphydryl Groups in Resting and Stimulated Rat Brain; Their Relationship with Protein Structure. (23729)

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The possibility that some structural rearrangement of cellular proteins is involved in excitation has been suggested by some early observations (reviewed by Hyden(1)) and by later work(2,3). In a recent publication(4), it was shown that electrical stimulation of frog and rat sciatic nerve and of cat, dog, and rat brain produces structural changes in proteins of these tissues, as indicated by the increase in the number of ionized side-groups. The present paper supplies further evidence in support of the hypothesis that excitation is accompanied by changes in protein configuration similar to those commonly associated with reversible denaturation.

Methods. Male albino rats (C. F. Wistar strain), weighing 250 to 350 g, were used. The skull was opened under topical ethyl chloride anesthesia and the exposed brain surface was kept moist with saline. Stimulation was effected through electrodes attached to the central end of the sciatic nerves by means of a 40 v. 60 cycle current from a Harvard stimulator. The brain was frozen by the procedure of Geiger *et al.*(5). Resting brain specimens were secured by the same procedure from rats rested at least 20 min. after exposure of the brain. The extracts were prepared according to the technic described previously(4). *Sulphydryl groups* were estimated by amperometric titration, using the technic of Benesch, Lardy, and Benesch(6). Results are expressed in terms of μM of $-\text{SH}$ per g of wet weight of brain.

The use of wet weight was judged satisfactory because there was no significant difference between the water content of resting and stimulated rat cortex: $79.2 \pm 0.9\%$ at rest and $79.5 \pm 0.4\%$ after 20 min. stimulation. When the $-\text{SH}$ determination was not done immediately after the experiment, the extract was kept frozen at -20°C . *Spectrophotometric estimation* of the ionization of protein side-groups was described in detail(4). It is based on the observation of Crammer and Neuberger(7) that at high pH the ultraviolet spectrum of proteins undergoes a shift in the wavelength of maximum absorption and an increase in extinction coefficient. The change is related to the number of ionized $-\text{OH}$ groups of tyrosine (in the 290-300 $\text{m}\mu$ range) and of $-\text{SH}$ groups of cysteine (in the 240-250 $\text{m}\mu$ range)(8). Results obtained with this method are expressed in terms of the side-group ionization ratio (SGIR) obtained by dividing the optical density of the extract at pH 12 by the optical density at pH 7.

Results and discussion. Table I summarizes the experiments with saline extracts of brain. It is seen that both the $-\text{SH}$ content and the SGIR are higher in extracts from stimulated brain than in those from resting brain. After stimulation of 1 min. the change is statistically not significant, but after 20 min. application of electrical current the differences are highly significant ($P < .001$) for both values. In previously published experiments(4), in rats

TABLE I. Changes in $-\text{SH}$ Groups and Side-Group Ionization Ratio (SGIR) in Resting, Stimulated, and Recovering Rat Brain.

| Stimulation | Recovery | $-\text{SH}^*$ | $\pm \text{S.D.}$ | SGIR† | $\pm \text{S.D.}$ | P | N |
|-------------|----------|----------------|-------------------|-------|-------------------|---------|----|
| Resting | | 4.56 | .57 | 1.80 | .08 | | 29 |
| 1 min. | | 5.08 | .78 | 1.95 | .07 | $>.05$ | 7 |
| 1 | 1 min. | 4.75 | .83 | 1.90 | .05 | | 6 |
| 1 | 2 | 4.15 | .56 | 1.80 | .05 | | 6 |
| 20 | | 6.55 | 1.02 | 2.09 | .11 | $<.001$ | 26 |
| 20 | 10 | 5.20 | .96 | 1.91 | .08 | | 6 |
| 20 | 20 | 4.17 | .32 | 1.85 | .04 | | 6 |

* In $\mu\text{M}/\text{g}$ wet wt.

† At 245 $\text{m}\mu$.

Brain extracts were made up in 0.15 M NaCl.

TABLE II. Changes in -SH Groups and Side-Group Ionization Ratio in Resting and Stimulated Rat Brain.

| Extract | | -SH† | ± S.D. | SGIR‡ | ± S.D. | P | N |
|------------------|-------------|------|--------|-------|--------|----------|---|
| Ringer sol. | Resting | 5.38 | .72 | 1.77 | .09 | | 8 |
| | Stimulated* | 8.90 | 1.21 | 2.05 | .10 | .01-.001 | 8 |
| Isotonic sucrose | Resting | 4.20 | .57 | 1.72 | .12 | | 7 |
| | Stimulated* | 6.01 | 1.23 | 1.95 | .07 | .01-.001 | 7 |

* 20 min. stimulation.

† $\mu\text{M/g}$ wet wt.‡ At 245 $m\mu$.

anesthetized with nembutal, SGIR values were lower (1.52 in resting brain and 1.85 after 20 min. stimulation). Table I also shows that the changes are reversible after removal of the stimulus.

When brain extracts were prepared in Ringer solution (without glucose), the SGIR changes were the same as in saline extracts. The -SH values, however, were found to be considerably higher (Table II). In extracts prepared in isotonic sucrose, both values were somewhat lower than in saline. Differences between resting and stimulated brain, however, were highly significant in all types of extracts tried.

The values given in Tables I and II represent the sum of free and bound -SH groups. To determine the bound -SH, extracts from resting and stimulated brain were dialyzed against 25 volumes of saline in a shaking dialyzer. At equilibrium, the -SH groups were titrated in the non-dialyzable fraction. Table III shows that about 75% of the -SH groups are bound to non-dialyzable molecules, presumably to proteins. The Table also shows that the changes produced by stimulation affect mostly the bound -SH. Stimulation causes a greater change in bound (55%) than in total (47%) or free -SH (29%).

Available evidence(8,4) suggests that the SGIR changes at 245 $m\mu$ indicate an increase in the number of ionized -SH groups of cysteine. Comparison of the direct estimation of titratable -SH groups with the SGIR has con-

firmed this assumption. Fig. 1 shows the correlation between the 2 sets of values, as obtained in 62 experiments with saline extracts of brain. The correlation coefficient ($r = 0.72 \pm 0.062$ S.D.) is highly significant ($P = <.001$). This correlation, however, is

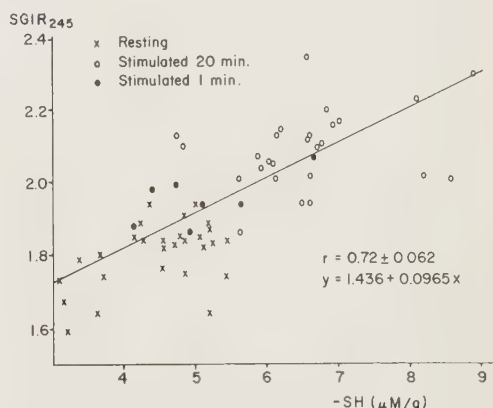


FIG. 1. Correlation between -SH groups and side-group ionization ratio. Abscissa: -SH groups in $\mu\text{M/g}$ wet wt; ordinate: side-group ionization ratio at 245 $m\mu$. Regression line calculated by least squares method. Significance of the correlation coefficient is $P = <.001$.

valid only in the given ionic environment. The relationship changes with the composition of the extraction medium. The influence of various electrolytes and pharmacological agents on the phenomena just described will be reported elsewhere.

It has been admitted that in native proteins only a fraction of the -SH groups present can be titrated directly. The increase in the

TABLE III. Changes in Free and Bound -SH Groups.

| | -SH ($\mu\text{M/g}$)* | | | | t | P |
|----------------|--------------------------|--------|-------------|--------|-----|-------|
| | Resting | ± S.D. | Stimulated† | ± S.D. | | |
| Total | 4.31 | .73 | 6.34 | .93 | 4.5 | <.001 |
| Non-dialyzable | 3.15 | .38 | 4.84 | .41 | 8.1 | <.001 |
| Dialyzable‡ | 1.16 | .41 | 1.50 | .69 | 1.1 | .3-.2 |

* Mean values of 8 extracts.

† 20 min. stimulation.

‡ Estimated by difference.

titratable -SH groups has been considered as a sign of denaturation. It seems, therefore, legitimate to interpret the changes just described as indications of a structural rearrangement of some protein molecules. Similar changes in -SH groups have been described in different systems; in the egg following fertilization(9) and in rhodopsin stimulated by light(10). The latter observation confirms Mirsky's hypothesis that light produces a reversible denaturation-like change in the protein moiety of visual purple(11). Other protein sidegroups are also "unmasked" in the process of denaturation, but the carboxyl or amino groups are not as readily detectable as the -SH groups. Ionization of the phenolic hydroxyl of tyrosine was described earlier(7,4).

The possible role of changes of protein configuration in the mechanism of cellular excitation is fully discussed elsewhere(12). Reversibility is one of the essential conditions for any change to be directly associated with excitation. The problem of the comparatively slow restoration of protein structure, as compared with the prompt return of excitability has to be approached with more sensitive methods, capable of detecting changes caused by a small number of impulses. Another problem, that of the relation between protein changes and the shift of Na^+ and K^+ ions during excitation, is under investigation.

Summary. An increase in titratable -SH

groups was observed in rat brain stimulated through its afferent nerves. The change is reversible when the resting state is restored. The increase produced by stimulation affects mainly the non-dialyzable, presumably protein-bound -SH groups. The change is interpreted as an indication of structural rearrangement of certain proteins which may participate in the mechanism of excitation.

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Pteroylglutamic Acid in Different Diseases. (23730)

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Sauberlich and Baumann(1) observed that pteroylglutamic acid (PGA) is converted to citrovorum factor (CF) in the body and the latter is the active form of the vitamin PGA. The metabolism of PGA has been studied in normal persons(2-4) and in patients suffering from liver diseases(5), pernicious anemia(6, 7), megaloblastic anemia(8), gout(9), cancer and tuberculosis(10). Considering the vari-

ous functions of PGA in the different physiological and pathological processes in the animal body it was of interest to study the metabolism of PGA in patients suffering from various diseases and in normal persons, by estimating urinary excretions of PGA and CF.

Methods. 17 cases of cirrhosis of liver, 10 of typhoid fever with positive blood culture,

TABLE I. 24-Hour Urinary Excretion of PGA and CF by Normal Subjects and by Patients Suffering from Various Diseases.

| | | | Supplements | | | |
|----------------------------|-----|-----------|----------------------|--|------------------------|--|
| | | Nil | 10 mg PGA (mouth) | 10 mg PGA + 500 mg ascorbic acid (mouth) | 10 mg PGA (intrav.) | |
| Normal (10) | PGA | 4.2 ± .1* | 4490 ± 137 | 4583 ± 147 | 7588 ± 62 | |
| | CF | .4 ± .0 | 14.0 ± .6 | 28.3 ± 1 | 14.1 ± .1 | |
| Cirrhosis of liver (17) | PGA | 2.7 ± .1 | 2611 ± 95 | 2662 ± 96 | 4837 ± 240 | |
| | CF | .2 ± .1 | 6.2 ± .3 | 12.2 ± .5 | 6.9 ± .2 | |
| Typhoid fever (10) | PGA | 1.4 ± .2 | 2984 ± 126 | 3128 ± 113 | 7450 ± 369 | |
| | CF | .3 ± .1 | 4.8 ± .3 | 10.1 ± .7 | 5.3 ± .4 | |
| Renal hypertension (10) | PGA | 3.5 ± .1 | 3342 ± 212 | 3440 ± 67 | 7540 ± 386 | |
| | CF | .1 ± .0 | 4.2 ± .2 | 7.0 ± .1 | 4.5 ± .3 | |
| Essential hypertension (5) | PGA | 4.0 ± .3 | 4492 ± 241 | | | |
| | CF | .4 ± .0 | 12.6 ± .3 | | | |
| Acute malaria (5) | PGA | 3.1 ± .3 | 4541 ± 450 | | | |
| | CF | .3 ± .0 | 4.6 ± .3 | | | |
| Influenza (5) | PGA | 3.0 ± .3 | 4697 ± 700 | | | |
| | CF | .3 ± .0 | 6.5 ± .8 | | | |
| Tuberculosis (5) | PGA | 4.3 ± .2 | 4458 ± 206 | | | |
| | CF | .4 ± .0 | 13.0 ± .9 | | | |
| Nutritional anemia (10) | PGA | 3.1 ± .2 | 3170 ± 128 | | 6140 ± 135 | |
| | CF | .2 ± .0 | 7.0 ± .3 | | 7.5 ± .4 | |

* Mean ± S.E.

Figures in parentheses indicate No. of subjects.

10 of renal hypertension, 5 of essential hypertension, 5 of acute malaria, 5 of influenza, 5 of tuberculosis and 10 of nutritional anemia were selected from patients admitted into the Nilratan Sircar Medical College Hospitals, Calcutta. Ten normal subjects selected for investigation were research students of Presidency College, Calcutta belonging to the same economic and social status as the selected patients. 24-hour output of urine of each subject was collected in bottles under toluene for 3 days consecutively. Each subject was then fed 10 mg PGA and urine was again collected for 3 days consecutively. PGA and CF excretions were determined in the different urine samples. When these excretions became normal the subjects were fed 10 mg PGA and 500 mg ascorbic acid. Urine samples were collected as before and excretions of PGA and CF determined. These excretions were also determined after intravenous injections of 10 mg PGA. PGA was estimated by the differential microbiological assay method(11) using *Streptococcus fecalis* R, CF was determined (1) using *Leuconostoc citrovorum* 8081 as test organism. The results are given in Table I.

Results. Cirrhosis of liver. Patients suffering from cirrhosis of liver excreted in urine lesser amounts of PGA and CF than normal

persons both before and after feeding of PGA. This is in agreement with the observations of Oji *et al.*(5). When PGA was injected intravenously CF excretions did not differ from the excretion after PGA was fed. This indicated that the diminished excretion of CF was not due to defective intestinal absorption of PGA. Nichol(12) showed that liver was the principal site of conversion of PGA to CF. In cirrhosis of liver the parenchymatous tissues are replaced by fibrous tissues. It is, therefore, possible that due to inadequate conversion of PGA to CF the excretion of the latter was diminished. Lesser excretion of PGA was possibly due to its destruction or its retention in the tissues.

Typhoid fever. Excretions of PGA and CF before and after feeding of PGA with or without ascorbic acid supplement also diminished in similar manner as the patients suffering from cirrhosis of liver. After intravenous injection of PGA excretion of CF did not increase as compared to excretion when PGA was fed. This indicated that although there was lesion in the small intestine this did not interfere with the absorption of PGA to CF (13). Less excretion of CF after PGA was fed might, therefore, be due to less conversion of PGA to CF in typhoid fever. The di-

minished excretion of PGA might be due to its destruction by the typhoid bacillus as it has been suggested(14) that pathological invasion of small intestine by bacteria might be responsible for the destruction of PGA. When PGA was administered intravenously so as to avoid the intestinal route, excretion of PGA was similar to excretions by normal subjects, which further supports the contention that PGA is destroyed in the intestine by the typhoid bacillus.

Renal hypertension. Patients suffering from renal hypertension also excreted diminished amounts of PGA and CF. Intravenous administration of PGA induced increased elimination of PGA as under similar conditions in normal persons but the excretion of CF did not increase as compared to that when PGA was fed. Kidney, therefore, might be responsible for the conversion of PGA to CF.

Acute malaria and influenza. Patients suffering from acute malaria and influenza excreted significantly less amounts of PGA and CF before administration of folic acid. When PGA was fed excretion of PGA was greater and the excretion of CF was less than the excretion by normal men. In these diseases, therefore, due to fever metabolic disturbances might interfere in the conversion of PGA to CF.

Nutritional anemia. In patients suffering from nutritional anemia the red blood cells were hyperchromic and macrocytic. These patients also excreted diminished amounts of PGA and CF both before and after PGA was fed. When PGA was injected intravenously while excretion of PGA was increased, excretion of CF did not change. Conversion of PGA to CF was, therefore, disturbed in these patients. Similar disturbance in PGA metabolism was also observed in pernicious anemia(6) and megaloblastic anemia(8).

The above observations indicate that the different tissues of the body are concerned in the conversion of PGA to CF, and in the diseased conditions studied, more PGA or preferably CF should be administered.

Summary. 1) Urinary excretions of PGA and CF were estimated in patients suffering from cirrhosis of liver, typhoid fever, renal hypertension, essential hypertension, acute

malaria, influenza, tuberculosis, nutritional anemia and in normal subjects. They were also studied after administration of PGA by both enteral and intravenous routes, with or without simultaneous administration of ascorbic acid. Patients suffering from cirrhosis of liver, typhoid fever, renal hypertension, acute malaria, influenza and nutritional anemia excreted diminished amounts of PGA and CF than normal persons. After feeding of PGA patients suffering from above diseases excreted less CF than normal persons under similar conditions. These excretions in patients suffering from essential hypertension and tuberculosis were similar to excretions by normal persons. Administration of ascorbic acid along with PGA enhanced urinary excretion of CF in all diseased conditions but the increase was less than that observed in normal subjects. Intravenous injection of PGA increased the output of PGA in urine but excretion of CF was similar to excretions observed after feeding PGA. 2) Different tissues of the body seem to be concerned in conversion of PGA to CF in the body and administration of PGA or CF in different diseased conditions is suggested.

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Occurrence of Porphyrins in Peripheral Nerves.* (23731)

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The occurrence of porphyrins in the central nervous system of a wide variety of animals was first described by Klüber(1). In his extensive survey of the nervous system of a large number of vertebrates, he(2) was able to demonstrate that the white matter of the central nervous system of warm-blooded animals contained coproporphyrin. Later, he extracted protoporphyrin in variable amounts. Klüber's spectroscopic examination of the peripheral nerves revealed that porphyrins were present in the optic, trigeminal, facial, and auditory nerves(1,2). He was unable, however, to detect the presence of porphyrins in the oculomotor group of nerves (III, IV, VI) or in spinal nerves. Further quantitative and qualitative study of the porphyrins in the central nervous system(3,4) confirmed Klüber's observations and it became apparent that the porphyrin in the central nervous system was a mixture of coproporphyrin and protoporphyrin. The discovery that the coproporphyrinuria in patients with poliomyelitis involved a type III porphyrin(7) focused attention on the coproporphyrin III concentration of the nervous system. Blanchard isolated and crystallized the coproporphyrin from beef brains and established that this was the type III isomer on the basis of the melting point of the methylester. It was early observed by one of us that the red fluorescence of the porphyrins in the nervous system was not uniformly distributed. Quantitative determination revealed a higher concen-

tration of coproporphyrin III in the medulla oblongata and brain stem than in cerebellum, cerebrum or spinal cord. Since porphyrin was abundant in the conduction areas of the central nervous system, and was found to be most concentrated in the important medullary region, it was postulated that the porphyrins may be involved in the process of conduction of nerve impulses. However, such a hypothesis would be untenable if porphyrins were absent in peripheral nerves. It was, therefore, decided to determine whether porphyrin was present in peripheral nerves and in what amounts. It was found that both protoporphyrin and coproporphyrin were relatively abundant in peripheral nerves. Moreover, it was observed that the peripheral nerves contain approximately 2 molecules of coproporphyrin to each molecule of protoporphyrin.

Methods and materials. The lumbo-sacral trunk and portions of the sciatic nerve were removed from the pigs immediately after they had been slaughtered. The nerves were transported to the laboratory in plastic bags packed in salted, crushed ice. They were separated from fat and blood vessels, washed in distilled water to remove blood, and either used immediately or wrapped in Saran and stored in the deep freeze. For each determination, 150 g quantities of nerves were homogenized in a blender with 500 ml of 4:1 ethyl acetate-glacial acetic acid for 20 to 30 minutes. The mixture was placed in the deep freeze until the temperature reached 6°C and was then filtered through a Büchner funnel. The filtercake was extracted with cold ethyl acetate-glacial acetic acid (4:1) until the filtrate became colorless. The porphyrins were

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TABLE I. Porphyrin Content of Peripheral Nerves of Pigs.

| | Coproporphyrin III/100 g ($\mu\text{g}/100\text{ g tissue}$) | Protoporphyrin/100 g | Coproporphyrin Protoporphyrin |
|-----|---|----------------------|----------------------------------|
| | 91.5 | 42.0 | 2.18 |
| | 96.0 | 47.3 | 2.03 |
| | 93.6 | 48.2 | 1.94 |
| | 99.0 | 44.6 | 2.21 |
| | 93.0 | 42.0 | 2.21 |
| Avg | 94.6 | 44.8 | 2.11 |

separated according to the method of Schwartz and Wikoff(6). Quantitative determinations of copro- and protoporphyrin concentrations were made fluorometrically(5).

Results. The results of 5 determinations are shown in Table I. It is apparent from this that the amounts of porphyrin in peripheral nerves are even more abundant than in the central nervous system. The porphyrins in peripheral nerves were found to be difficult to demonstrate spectroscopically, but the emission bands of porphyrins in peripheral nerves were observed with a Gaertner quartz spectrograph.

The dominant porphyrin in peripheral nerves as in the central nervous system was

coproporphyrin III. It was noted, however, that there was a constant ratio of coproporphyrin to protoporphyrin when the nerve tissue was protected from autolysis before the determination.

Summary. Both coproporphyrin III and protoporphyrin were determined in the peripheral nerves of pigs. An average of 5 determinations showed that 100 g of nerve contained 94 μg of coproporphyrin and 45 μg of protoporphyrin. The ratio of coproporphyrin to protoporphyrin was approximately 2:1 and appeared to be constant.

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Absence of Gastro-Intestinal Lesions in Rats Following Ingestion of Silica. (23732)

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The finding of solid silica in lungs and other tissues and its toxicity when inhaled or injected intravenously has led to attempts to produce lesions by ingestion. (Piezoelectric effects of crystalline silica have been blamed but no piezoelectric effect is possible unless the crystal is compressed in one direction.) Silica occurs in food and to the extent of 2% in human blood ash. We wished to determine whether 10% of the diet as silica dust would be toxic. Seventy weanling rats were divided into 7 groups of 10 rats each. One group was fed Purina dog chow and the others 90% Purina dog chow and 10% of one of the forms of silica shown in Table I.

The rats were fed these diets 3 months and then killed and histological preparations made of the stomach, small and large intestine, liver, spleen, pancreas, adrenals, mesenteric lymph nodes and lung. No lesions were found in any of the organs studied.

It may be objected that 3 months is not sufficient time to produce lesions with silica. Clinical symptoms of silicosis have been reported in humans breathing silica dust for 6 months but most cases studied have been exposed to silica dust from 1 to 40 years. If the rat is to be used as an experimental animal it must be assumed that it lives more rapidly and that changes occur in less time than in

TABLE I.

| Name | Particle size in μ |
|---|---------------------------|
| Linde silica | .02-.05 |
| Columbia-Southern Hi-Sil 101 | .03 |
| Dow Corning silica (unpelletized) | .01-.02 |
| Powdered silica gel | |
| Sodium metasilicate | |
| Carborundum Co. Fibrofrax (silica fibers) | |

humans.

Summary. Powdered silica or Fibrofrax

mixed with the rat diet to the extent of 10%, produces no tissue pathology in the gastrointestinal tract, liver, spleen, pancreas, adrenals, lung or mesenteric lymph nodes when fed continuously for 3 months.

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Cerebrospinal Fluid Enzymes in Central Nervous System Lipidoses* (with particular reference to Amaurotic Family Idiocy) (23733)

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The diagnostic usefulness of certain enzyme levels in cerebrospinal fluid (CSF) and serum derived from cases of central nervous system (CNS) disease has recently been explored (1-5). Various authors have demonstrated transient glutamic oxalacetic transaminase (GOT) elevation in CSF derived from clinically determined or experimentally induced cerebral infarction(1,2). Other investigators, in evaluating the utility of CSF GOT in a broad spectrum of neurologic disorders, were unable to report any conclusive alterations in the levels of this enzyme(4).

Significant rises in serum GOT and aldolase have recently been recorded in cases of Infantile Amaurotic Family Idiocy (IAFI) (5). These findings indicating that a number of determinable serum enzymes is increased in one of the CNS lipidoses led to an assessment of their activity within the CSF of cases in this disease category. Concurrent serum levels were also determined to clarify any possible correlation between the two biological fluids. The activity of lactic dehydrogenase (LD) was also measured in the serum and CSF of selected lipidosis and control cases.

Material and methods. a.) Clinical: Re-

peated serum and CSF specimens from 9 patients with IAFI (at varying stages of the illness) and 2 cases of Niemann-Pick's Disease (NPD) with CNS involvement were studied in regard to GOT, aldolase and LD levels. Similar enzyme studies were performed on 33 additional children afflicted with other neurological disorders. b.) Experimental: 10 mongrel dogs, each weighing about 13 kg, were injected intramuscularly with 1 g (GOT enzymatic activity: 80,000 u./g) of fresh heterologous canine grey matter. An additional 3 dogs were inoculated with a similar homogenate previously incubated at 100°C for 1½ hours (GOT enzymatic activity, 0.0 u./g). All dogs were then sequentially studied by daily serum GOT determinations for periods up to 8 days. The method employed for determination of serum and CSF GOT was that of Karmen, Wróblewski and LaDue(6) based on spectrophotometric measurement in the near ultraviolet with the Beckman D.U. spectrophotometer. Serum and CSF aldolase activity was estimated with the colorimetric procedure of Sibley and Lehninger(7). The LD activity of the serum and CSF samples were also determined spectrophotometrically at 340 m μ with the Beckman D.U. using the method of Wróblewski and LaDue(8).

* This project was performed under auspices of Nat. Tay-Sachs Assn.

TABLE I. Enzyme Data in Serum and CSF from Cases of CNS Lipidosis and Other Neurologic Disorders.

| Diagnosis | No. of patients | Age | Serum | | | | |
|---------------------------|-----------------|-------------|---------------|----------|--------------------|-----------|--------------------|
| | | | GOT, units/ml | | Aldolase, units/ml | | LD, units/ml |
| Amaurotic family idiocy | 9 | 9-14 mo* | 127 (2)† | 123-130‡ | 10.1 (2) | 7.7-12.4 | 1641 (4) 1362-1942 |
| | | 15-24 " * | 92 (17) | 55-152 | 8.9 (17) | 5.4-15.7 | |
| | | 25+ " * | 65 (5) | 41- 91 | 9.2 (5) | 5.8-12.8 | |
| Niemann-Pick's disease | 2 | 10-22 " | 170 (8) | 91-248 | 9.3 (8) | 6.9-12.5 | 576 (2) 504- 648 |
| Cerebral palsy | 15 | 13 mo-12 yr | 39 (20) | 20- 64 | 9.1 (20) | 2.8-15.6 | 700 (3) 552- 870 |
| C.N.S. anomaly | 13 | 1-9 yr | 36 (19) | 20- 51 | 7.1 (19) | 4.0-11.5 | 719 (2) 714- 724 |
| Obstructive hydrocephalus | 4 | 9 mo-9 yr | 45 (2) | 44- 46 | 15.1 (2) | 13.9-16.5 | |
| Craniopharyngioma | 1 | 4 yr | 50 (3) | 37- 58 | 10.2 (3) | 6.6-15.2 | |
| Normal range | | | | 22- 40 | | 5-10 | 200- 680 |
| CSF | | | | | | | |
| Amaurotic family idiocy | 9 | 9-14 mo* | 72 (2) | 50-94 | 3.6 (2) | | 143 (4) 130-152 |
| | | 15-24 " * | 38 (17) | 22-60 | 1.4 (17) | .3-2.7 | |
| | | 25+ " * | 33 (5) | 24-47 | .9 (5) | .1-2.2 | |
| Niemann-Pick's disease | 2 | 10-22 " | 32 (4) | 25-41 | 4.6 (4) | 2.3-7.4 | 75 (1) |
| Cerebral palsy | 15 | 13 mo-12 yr | 10 (20) | 5-18 | .4 (20) | .1-1.0 | 25 (3) 20- 33 |
| C.N.S. anomaly | 13 | 1-9 yr | 11 (19) | 4-19 | .5 (19) | .0-1.1 | 41 (2) 39- 43 |
| Obstructive hydrocephalus | 4 | 9 mo-9 yr | 33 (5) | 24-41 | 2.2 (4) | 1.1-5.0 | |
| Craniopharyngioma | 1 | 4 yr | 19 (3) | 12-34 | 1.1 (3) | 1.0-1.3 | |
| Normal range | | | | 4-14 | | 0-1 | 5-40 |

* Criteria for phases of disease given elsewhere(9).

† Arithmetic mean and, in parentheses, No. of separate determinations.

‡ Range of determined values.

Results. The serum and CSF GOT were invariably elevated in all cases of IAFI studied (Table I). The enzyme levels were higher in the initial phases of the disease. With further progress, the abnormal values diminished, but even in the protracted aspect of the disorder (beyond 2 years of illness), the average serum and CSF GOT values were well above the upper limits of normal. A distinct correlation between the simultaneously determined levels in the 2 biologic fluids was apparent. The serum and CSF GOT were considerably increased in NPD. Only one patient in the control group showed any consistent serum GOT rise (an 8 months old male child with severe progressive cerebral disease of unknown etiology). The same infant also showed an elevated CSF GOT. Three children, all with continuing obstructive hydrocephalus, demonstrated normal serum GOT but consistent elevations of CSF GOT. The CSF GOT in 1 case of confirmed craniopharyngioma was increased.

CSF aldolase was usually elevated in the 11 cases of CNS lipidosis (IAFI and NPD). A

comparable increase was also noted in the children with hydrocephalus described above. The corresponding serum aldolase values in all groups were within the normal range.

LD levels (both in serum and CSF) were notably increased in the lipidoses, while they were approximately normal in the limited number of control cases studied (Table I).

In the dogs inoculated intramuscularly with grey matter emulsion, an approximately 2-fold rise in serum GOT was seen in the 2 days subsequent to injection. No such rise was noted in dogs injected with an inactivated cerebral homogenate or mesenteric fat administered in similar volume (Table II).

Discussion. It has been reported previously that serum GOT and aldolase are increased in cases of IAFI and NPD(5). The transient rise in serum aldolase was thought to be caused principally by the coexistent neuromuscular atrophy. Since myelopathic muscle atrophy has not been equated with any rise in serum GOT(4) and since no structural abnormality other than ganglionic necrosis has been identified in IAFI, it was provision-

TABLE II. Serial Serum—GO Transaminase Values (Units/ml) in 13 Dogs following Intramuscular Injection of Fresh or Heat-Inactivated Brain Grey Matter.

| Inoculum | No. of dogs | Fasting before inoc. | Time after intramusc. inoc. | | | | | |
|-----------------------------------|-------------|----------------------|-----------------------------|---------|---------|---------|---------|---------|
| | | | 5 hr | 1 day | 2 days | 3 days | 4 days | 7 days |
| Grey matter, 1 g | 10 | 35 (20)* | 55 (10) | 64 (17) | 62 (14) | 44 (10) | 39 (10) | 38 (10) |
| | | 27-44 † | 44-74 | 40-107 | 28-166 | 28-68 | 18-70 | 28-67 |
| Heat inactivated grey matter, 1 g | 3 | 31 (6) | 35 (3) | 30 (6) | 39 (4) | 27 (3) | 30 (3) | 31 (3) |
| | | 24-41 | 32-40 | 20-53 | 25-64 | 22-35 | 26-35 | 30-32 |

* Arithmetic mean and, in parentheses, No. of separate determinations.

† Range of determined values.

ally suggested that the sustained hypertransaminasemia was attributable to degeneration of brain, a tissue containing high concentrations of GOT. Significant quantities of CSF GOT were shown in all cases of CNS lipidosis regardless of disease stage, presumably due to enzymatic liberation. This suggests that the GOT elevations in both CSF and serum were the result of a single source of enzyme release. An appreciable but short-lived rise in serum GOT was demonstrated in dogs injected with fresh grey matter emulsion.

It would seem, therefore, that any disease process resulting in cerebral necrobiosis can cause an increase in CSF GOT levels, and if sufficiently pronounced, a rise in serum GOT content. The resultant enzyme elevation is also a guide to any prolongation of necrosis. Thus, if the brain damage is episodic (*i.e.*, cerebral vascular disease), the GOT rise will be transient. If, on the other hand, the nature of the underlying disorder causes a continuing breakdown of neural tissue, such as in CNS lipidosis(9), the secondary GOT elevation will be more enduring. Paranatal brain injury does not appear to cause any rise in CSF GOT, if the determinations are carried out years after the sustained damage. On the other hand, rapidly developing and progressive hydrocephalus with cerebral degeneration has resulted in a rise of CSF GOT.

Some have postulated that the blood-brain barrier causes a failure of correlation between serum and CSF GOT. Cases of hepatic diseases with hypertransaminasemia and normal CSF GOT levels are offered as supportive evidence(2). The close correlation of GOT enzyme content in the two fluids in cases of IAFI may merely reflect extensive and progressive neural degeneration liberating such amounts as to overcome any presumed thresh-

old created by the blood-brain barrier.

Some investigators have indicated the lack of any abnormal enzyme findings in CNS degenerative disorders(4). No specification of the type of disturbance was noted in these reports. Since an elevated GOT content of CSF seems to be an indication of actively progressive cerebral necrosis rather than a measurement of previously wrought degeneration, it is conceivable that the quiescent phase of a formerly fulminant disease would not show any abnormal GOT findings. The CSF aldolase elevation particularly in the early years of IAFI is probably also explained by continuing destruction of enzyme-bearing neural tissues.

Wróblewski and coworkers have noted that LD activity is increased in the CSF from cases of CNS carcinomatosis, leukemia and lymphoma, but not in patients with primary brain tumor or degenerative CNS disease(10). Application of this procedure to a small number of cases of CNS lipidosis has revealed a significant rise in enzyme activity suggesting that an even wider range of intracellular enzymes are liberated following brain tissue destruction.

Summary. 1) Simultaneous determinations of glutamic oxalacetic transaminase (GOT), aldolase and lactic dehydrogenase (LD) were estimated of sera and cerebrospinal fluid (CSF) derived from 9 cases of infantile Amaurotic Family Idiocy (IAFI), 2 cases of Niemann-Pick's Disease (NPD) and 33 control patients with other neurologic disorders. 2) Parallel values of GOT and LD were invariably elevated in IAFI, while only the serum and CSF GOT were increased in NPD. The CSF aldolase was usually elevated in all cases of CNS lipidosis, while serum aldolase levels were but slightly increased. Significant

CSF enzyme rises were also noted in occasional control patients with actively progressive cerebral degeneration. 3) The data suggest that nervous system necrosis incident to the lipidoses may be biochemically distinguished from other forms of neural degeneration by the analysis of multiple enzyme levels in serum and CSF.

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Dermal Fibrosis Following Subcutaneous Injections of Serotonin Creatinine Sulphate.* (23734)

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(Introduced by Sidney Farber)

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5-Hydroxytryptamine (serotonin) has been associated with many physiologic and disease states, but its primary function is at present unknown. One of its most dramatic associations is in patients with the "carcinoid syndrome," in which there are several or all of an unusual group of findings: metastatic carcinoid tumor (argentaffinoma) of the gastrointestinal tract, dyspnea, diarrhea, episodes of flushing and cyanosis, hepatomegaly, ascites and edema, peptic ulcers, and cardiac lesions, chiefly affecting the pulmonic and tricuspid valves. Administration of serotonin to experimental animals has been shown to reproduce the clinical features of the syndrome, and intraperitoneal injections of the serotonin precursor, 5-hydroxytryptophane, have been followed by gastric mucosal erosions(1). It has been hypothesized that the cardiac valvular lesions in the carcinoid syndrome, which consist chiefly of fibrous tissue, are caused by

serotonin, and it has been postulated that serotonin elicits a fibrous proliferation of the cardiac valves(2). Up to the present time, however, there has been no experimental evidence to support this hypothesis. In the present study, twice daily subcutaneous injections of serotonin creatinine sulphate resulted in a collagenous and fibrous proliferation in the dermis of rats, with hyperplastic changes of the epidermis. Similar changes were not observed in control animals injected with physiologic saline and with carbon tetrachloride.

Materials and methods. Fifty-three rats of the Sprague-Dawley strain[†] were injected subcutaneously twice daily for periods of up to 342 consecutive days, using 8 mg doses of

[†] Purchased from the Charles River Breeding Laboratories, Cambridge, Mass.

[‡] Kindly supplied, in part, by Dr. George Berryman of Abbott Laboratories, North Chicago, Ill., and purchased from the California Fdn. for Biochemical Research, Los Angeles.

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FIG. 1. The rat below received subcut. injections of serotonin creatinine sulphate 72 consecutive days; the rat above received similar injections of physiologic saline. Note focal scarring and loss of hair of skin of animal that received serotonin. Necrosis of digits and tip of tail is also apparent.

serotonin creatinine sulphate[†] dissolved in one milliliter of physiologic saline. The skin of the back was used as the injection site. Groups of animals were 59 and 67 days of age at the time injections were begun. Males and females were used in different experiments. Twenty-four control animals received twice daily injections of one milliliter of physiologic saline for periods of up to 342 consecutive days, and 12 additional control animals received 0.2 ml of carbon tetrachloride twice weekly for 5 months. Animals were sacrificed at approximately monthly intervals in order to study the developmental stages of the lesions. Fixation of tissues was in Zenker's solution, and in neutral buffered 10% formalin. Histologic sections were stained with phloxine methylene blue, hematoxylin and phloxine, periodic acid Schiff reagent, elastica-Van Gieson connective tissue stain, and a modification of Bielschowsky's silver stain for reticulum.

Results. After approximately 30 days the animals receiving serotonin lost most of their hair in the regions of the injections, (Fig. 1), and their skin became visibly thickened and later cornified. Diffuse and constant reddening

of the scarred areas became apparent after approximately 4 months. With continuation of the injections the changes became progressively more marked. Skin in areas away from the injection sites was normal; in the affected areas, the histologic changes were as described below.

After approximately 30 days the dermal collagenous tissue in the regions about the injection sites became dense, was arranged in compact, closely packed bundles, and took an eosinophilic stain (Fig. 3). There was little or no inflammatory cell reaction, and no apparent increase in reticulum fibers as demonstrated with the reticulum stain. With the periodic acid Schiff reagent, many positively staining collagenous bands were evident within the dermis; similarly stained areas were not found in sections of skin from control animals. The staining with periodic acid Schiff reagent was interpreted to represent immature collagen fibers, probably due to new fiber formation. A marked increase in the number and

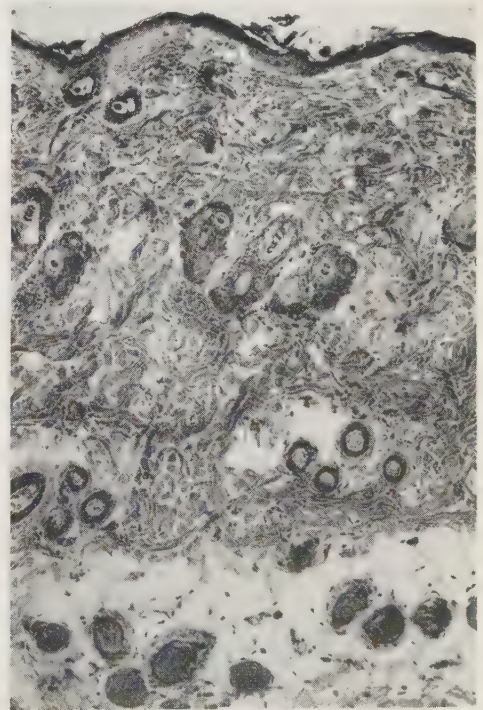


FIG. 2. Skin from an injection site of animal in Fig. 1 that received only physiologic saline 72 days. Note loose collagenous bundles in dermis, abundant hair follicles, and thin epidermis. PMB (phloxine methylene blue) $\times 67$.

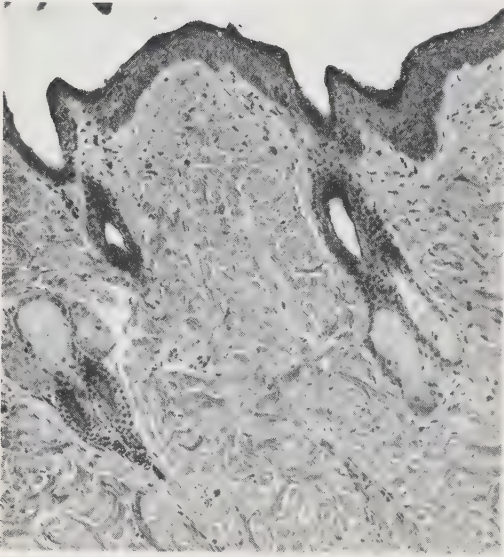


FIG. 3. Skin from injection site of animal in Fig. 1 that received serotonin creatinine sulphate 72 days. Note dense, closely packed collagen bundles in thickened dermis, decrease in hair follicles, and thickening of epidermis. PMB (phloxine methylene blue) $\times 67$.

size of small blood vessels of capillary dimensions was noticeable within the dermis, and the epidermis showed hyperplasia, with thickening due to proliferation of cells (Fig. 3, 4). These changes became marked at approximately 60 days. Hair follicles decreased in number and sebaceous glands became larger than normal and were located deep within the dermis.

After approximately 6 months, the dermis became the site of fibroblastic proliferation and increased vascularity, and small arterioles in the basal regions of the dermis showed an eosinophilic staining with the phloxine methylene blue and hematoxylin and phloxine stains, resembling the changes found in small arterioles in benign nephrosclerosis. The basal layer of the epidermis frequently showed proliferation of basal cells to form small nests within the dermis, with anaplasia, suggesting "pre-cancerous" lesions (Fig. 4). Hair

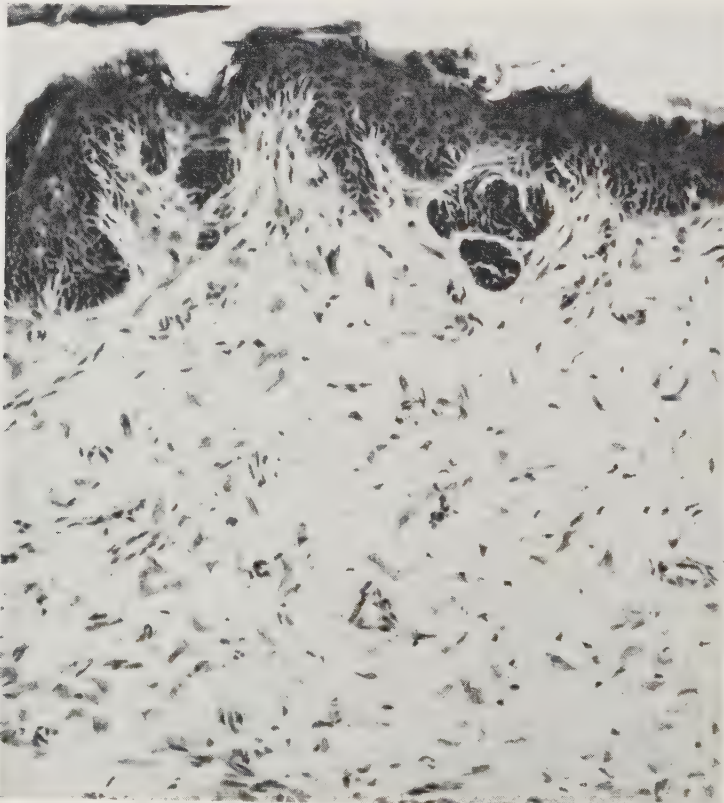


FIG. 4. Skin from injection site of animal that received serotonin creatinine sulphate 342 consecutive days. Note abundance of fibroblasts and increased vascular channels in thickened dermis, thickened epidermis with small islands of basal cells in adjacent dermis, and absence of hair follicles and sebaceous glands. PMB (phloxine methylene blue) $\times 400$.

follicles and sebaceous glands became infrequent or absent in affected areas (Fig. 4). The above changes occurred in varying degree in all animals injected with serotonin, and no sex difference was noted. Control animals did not show similar changes after 342 days of saline injections or after 5 months of carbon tetrachloride injections.

Discussion. The present study indicates that serotonin creatinine sulphate stimulates a proliferation of collagenous and fibrous tissue within the dermis at the site of local injections. The manner in which this occurs is at present unknown, and further studies are in progress in an attempt to determine whether other substances closely related to serotonin can cause a similar fibrous proliferation of the dermis. The absence of significant inflammatory cell response preceding the collagenous and fibrous proliferation is a feature of interest and one that deserves further investigation. Fibrous proliferation was not observed in visceral and other organs of the animals, despite careful and complete autopsy search. This may have been due to a rapid inactivation of the injected serotonin by the mono amine oxidase system present in many tissues of the body(3). The hypothe-

sis that serotonin causes cardiac valvular fibrous proliferation in patients with the carcinoid syndrome would appear to receive some support from the present study.

Summary. 1) Serotonin creatinine sulphate was injected subcutaneously twice daily into rats in 8 mg doses for periods to 342 consecutive days. After approximately 30 days of injections there was observed a progressive collagenous and fibrous proliferation within the dermis, an increased vascularity, hyperplasia of the epidermis, vascular changes in arterioles of the dermis, and diminution in skin appendages, all at sites of serotonin injections. Control injections of physiologic saline and carbon tetrachloride did not result in similar changes. 2) The present findings appear to lend some support to the hypothesis that serotonin causes the cardiac valvular fibrosis observed in patients with the carcinoid syndrome.

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Influence of H³- β -Sitosterol on Sterol Excretion.* (23735)

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It was previously observed in these laboratories(1,2) that soybean sterols inhibit the increase in blood cholesterol of rats receiving a high cholesterol diet. Also, plant sterols were found to be esterified *in vitro* by pancreatic cholesterol esterase under the same conditions as cholesterol, albeit, at a slower rate than cholesterol. It was considered that soybean sterols inhibit cholesterol absorption by

competing with cholesterol for the esterifying cholesterol esterase system. Bisset and Cook (4) fed humans 10 g of sitosterol a day and found that total fecal sterol increased, but only to about 60% of that fed. There was also an increased excretion of esterified sterol and a fraction rich in sitosterol esters was isolated by chromatography.

The experiment described below was designed to give more direct information on the absorption of sitosterol than previously obtained(3) and to allow measurement of the

* This study was supported in part by grants from Am. Heart Assn., P.H.S., and Life Insurance Medical Research Fund.

TABLE I. Fecal Sterol Excretion following the Feeding of H³- β -Sitosterol. 7 rats/series.

| Group* | Fecal sterols total, mg | Fecal H ³ - β -sitosterol, mg | Fecal sterol other, mg | Specific activity† total fecal sterol, μ c/mg |
|--------------------------------------|-------------------------|--|------------------------|---|
| H ³ - β -sitosterol | 22.9 \pm 3.5† | 11.8 \pm 1.6 | 11.1 \pm 2.5 | .042 \pm .004 |
| Control | 6.0 \pm .4 | | 6.0 \pm .4 | |

* Represents 24-hr excretion period.
specific activity of .081 μ c/mg.

† Each animal received 25 mg H³- β -sitosterol with
S.E. of mean.

excretion of cholesterol and related sterols. To overcome the difficulties of determining cholesterol in the presence of sitosterol, H³- β -sitosterol was fed in a cholesterol-free mixture; total fecal sterols as well as the amount of H³- β -sitosterol excreted were determined.

Methods and materials. The tritium labeled β -sitosterol was prepared by means of a platinum-catalyzed exchange reaction between β -sitosterol and a mixture of tritium acetic acid, tritium water and tritium gas as described by Fukushima and Gallagher(5). Two groups of male rats weighing 225-250 g, raised on Purina pellet chow, were used. One group, after being fasted 24 hours, received, by intubation, an emulsion containing 25 mg (2.04 μ c) H³- β -sitosterol, 50 mg egg albumin, 70 mg sodium taurocholate, and 75 mg oleic acid made up in 3 ml of saline. The other group received either the emulsion without H³- β -sitosterol or a sterol-free diet prepared as described previously(3). The sterol excretion was the same in the control animals whether the sterol-free emulsion or diet was administered. At the end of 24 hours the animals were sacrificed, the intestinal tract removed, and washed with 50 ml of saline. The washings from the intestine were pooled with the feces. Non-saponifiable lipid extracts were prepared according to procedures described earlier(3). The sterol content was determined gravimetrically as the digitonide (3). The tritium activity of the fecal sterols was determined as follows: fecal sterol digitonides were combusted in oxygen to water and CO₂; the water was reduced to hydrogen gas by heating with Zn dust for two hours at 420°C. The hydrogen gas was liberated in an ionization chamber and its tritium activity determined. A galvanometer drift of 1 mm/sec was equal to 2×10^{-3} μ c of tritium with an error of $\pm .1$ mm/sec or 2×10^{-4} μ c.

Results. Table I shows that the feeding of

25 mg of H³- β -sitosterol gave, during the following 24-hour period, 22.9 mg of total fecal sterol. Based on the specific activity of the sitosterol fed (.081 μ c/mg) 11.8 mg of this amount was H³- β -sitosterol and 11.1 mg other sterol. Calculation of the amount of β -sitosterol absorbed after subtracting total fecal sterol minus the control excretion gave a value of 8.1 mg or 32.4% of that fed. On the other hand, calculation based on the amount of fecal H³- β -sitosterol showed 13.2 mg or 52.8% of that fed was unaccounted for and presumably absorbed. During the period of H³- β -sitosterol administration there was a significant increase ($P < .05$) in fecal excretion of sterols other than β -sitosterol from 6.0 to 11 mg/day.

Discussion. It is evident from the data that after administration of β -sitosterol there was a considerable increase in the fecal excretion of cholesterol and other sterols which would not be apparent from chemical balance data alone. Likewise, there was a greater absorption of β -sitosterol than would be assumed from the chemical data. This confirms our earlier report(3) of significant plant sterol absorption under the optimum conditions formulated for cholesterol absorption. Plant sterol absorption ranging from 10 to 60% of that fed has also been reported by other investigators(6-9). The increased excretion of endogenous cholesterol when β -sitosterol is fed is in accord with the suggestion(1) that plant sterols inhibit the increase in blood cholesterol levels due to high cholesterol diets by competing with cholesterol for the sterol absorptive system of the intestinal wall. It should be pointed out that while β -sitosterol may inhibit the reabsorption of endogenous cholesterol, it does not necessarily follow that it will decrease normal blood cholesterol levels, since we have recently shown (10) that removal of reabsorbed endogenous

cholesterol *via* a thoracic lymph fistula produced an 8-10 fold increase in liver cholesterol synthesis from 1-C^{14} -acetate.

Summary. An increased fecal excretion of cholesterol and related sterols followed the administration of H^3 - β -sitosterol to rats receiving cholesterol-free emulsions containing bile salt and fatty acid. Approximately 53% of the administered labeled β -sitosterol was not recovered in the feces. Our study provides additional evidence that plant sterols are absorbed in the same manner as cholesterol and thereby compete with cholesterol for the sterol absorptive mechanism.

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Effects of Type of Restraint upon Heat Tolerance in Monkeys.* (23736)

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In the course of other experiments, monkeys were exposed to a warm environment while restrained on an animal board of the type ordinarily used for dogs and cats. Under these conditions, the rectal temperatures increased to an undesirable degree. The monkeys were able to reach equilibrium at an acceptable rectal temperature, however; with a different method of restraint; here the arms were held at the side of the body and the head was supported by a yoke around the neck. The observations are reported to call attention to the sensitivity of the temperature regulating mechanism of these animals to slight changes in the method of restraint.

Material and methods. Thirteen Macaca monkeys of both sexes ranging in weight from 3 to 6 kg, were used in 18 tests. Of the 6 exposed at $29^\circ\text{C} \pm 2^\circ$, the temperature of the animal quarters, 3 monkeys were tested with

each type of restraint; heart rates were recorded on 2 of the 3. Two of the other monkeys were also exposed at 38°C , one with each type of restraint. The remaining 7 monkeys were exposed once at 38°C . This temperature was controlled to $\pm 0.5^\circ\text{C}$ and the relative humidity was $45\% \pm 2\%$. The critical features of the 2 types of restraint, both with animal in prone position, are as follows: *Arms forward*: The animal rested with head free on a solid wooden platform, tied by wrists and ankles, with arms extended beyond the head. The knees and elbows had little freedom to bend. *Arms backward*: The animal rested on a platform of 0.5 in. wire mesh with an air space of 1.5 in. between it and the table. The body was held in position by yoke around the neck and by tying the arms at wrist and elbow along the side of the body. The legs were tied at the ankles but were only held loosely. At 29°C rectal temperatures were recorded with a Yellow Springs Tele-Thermometer thermistor and heart rate was obtained with a Burdick Electrocardiograph. At 38°C a cop-

* Supported in part by Central Scientific Fund, State Univ. of Iowa.

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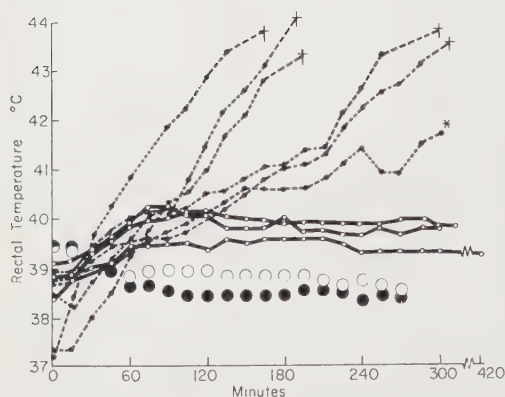


FIG. 1. Course of rectal temperature during restraint. Individual monkeys at environmental temperature of 38°C are shown by dashed lines in "arms forward" position and by solid lines in "arms back" position. Group averages at environmental temp. of 29°C are shown by closed circles in "arms forward" position and by open circles in "arms back" position. Mean rectal temp. and range of variation during entire period of restraint for individuals at 29°C were: in "arms forward" position, $37.4 \pm .5$, $37.8 \pm .7$, 39.2 ± 1 , $38.5 \pm .4$ and $38.7 \pm .5$; in "arms back" position, $37.6 \pm .9$, $39.2 \pm .4$, $39.6 \pm .2$ and $38.8 \pm .7$ °C.

per constantan thermocouple and a Leeds and Northrop Speedomax recorder were used to obtain the rectal temperature. The thermocouple or thermistor was inserted to a depth of 6 to 8 cm and recorded to the nearest 0.05°C.

Results. In nearly 5 hours at 29°C the type of restraint had a negligible effect on rectal temperature and heart rate. In Fig. 1, the mean temperature declined in the first hour and then remained nearly constant through the fourth hour. The mean heart rate for the entire experiment was 255 beats per minute for the "arms back" restraint and 254 beats per minute for the "arms forward" restraint. The range of the heart rate during this time was from 210 to 290 beats per minute.

At 38° the rectal temperatures of individual monkeys are shown in Fig. 1. With "arms back" restraint the temperature increased about 1°C in the first hour and then remained nearly constant through the sixth hour. These monkeys maintained a fresh and active attitude throughout this period. With "arms forward" restraint, the temperature rose progressively and the monkeys became

unresponsive after about 2 hours.[†]

Discussion. The ability of the restrained monkey to maintain thermal equilibrium under heat stress, appears to depend on differences in the method of restraint that have not been identified. Two possibilities may be mentioned; one, that the raised wire-mesh screen in the "arms back" restraint favored heat loss by evaporation; the other that the "arms forward" method was less acceptable to the animals with the result that they struggled more, increasing the heat output, or through an emotional response, restricted the peripheral circulation, decreasing the sensible heat loss. That these monkeys do not take kindly to restraint is indicated by the elevation in heart rate at 29°C above what has been reported(1) under "resting conditions" (average 192, range 165-240), and by the hyperglycemia reported by Poirier *et al.*(2). The lability of the vasomotor system to strong emotion has been recognized at least since the work of Hill(3). Whatever the cause, it is evident that the ability of these animals to withstand heat stress is critically affected by the method of restraint.

Summary. Macaca monkeys attached to an animal board by means of the wrists and ankles with arms and legs extended, in an environment of 38°C and 45% relative humidity, were unable to maintain their temperature equilibrium. The rectal temperature increased steadily to over 43°C in a period of from 3 to 5 hours. When restrained on a wire mesh platform by means of a neck yoke and attachment of the arms along the side of the body, the monkeys held a steady rectal temperature of about 40°C for 5 hours. At a room temperature of 29°C equilibrium of rectal temperature was maintained with both types of restraint with average heart rates over a period of 4.5 hours of 254 for one type and 255 for the other. It is concluded that the manner in which a monkey is restrained can modify its physiological responses during exposure to heat stress.

[†] The monkey whose record in Fig. 1 is marked with an asterisk was petted and given water to drink during exposure.

the generous loan of some of the animals used in this study, and M. A. Folk for the illustration.

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Viability of Monkey Kidney Tissue Cultures Stored at 5°C. (23737)

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The widespread use of monkey kidney cells for production and study of mammalian viruses has created a demand for this animal which is becoming difficult to meet. Prolongation of the useful life of tissue cultures would be one measure to alleviate this situation. Monkey kidney epithelial cells generally can be maintained in a usable condition for only 3-4 weeks at 37°C. This period has been extended somewhat by storing cultures at room temperature. An early report by Fischer(1) described such a procedure for the storage of chick tissue cultures. In addition to extending the life of monkey kidney tissue cultures, there is an obvious advantage in studying a small number of cultures for non-specific degeneration, foamy, and simian agents, before the remainder of the cultures are used for vaccine safety testing or for virus research purposes.

It has been observed that storage of monkey tissue cultures at 4-6°C provides a satisfactory procedure for relatively short-term preservation of large numbers of cultures. The following report describes such observations along with studies of the sensitivity of stored cultures to poliovirus.

Methods. Kidneys were removed from exsanguinated 5-7 pound rhesus monkeys and trypsinized as generally outlined by Youngner (2). The cortical tissue from 4 sets of kidneys was minced, pooled, and washed 3-4 times in 3 volumes of Earle's salt solution containing penicillin and streptomycin (100 units and 100 µg, respectively). The washed tis-

sue was trypsinized with prewarmed (37°C) 0.25% trypsin in Earle's salt solution in a blender jar for 10-minute intervals at a speed just below the foaming level of the trypsin. Usually 4 extractions were made with 20-25 ml of trypsin solution per kidney decanted and replaced at each interval. The trypsinized cells were pooled and centrifuged for 15 minutes at 10 g, following which they were washed 3 times in Earle's salt solution containing 2% calf serum and centrifuged for 10 minutes after each wash. The cells were transferred to a calibrated conical centrifuge tube and centrifuged at 10 g for 3-4 minutes after which they were ready for resuspending in nutrient media. Medium 199(3) was prepared by diluting a 10 X concentrate with sterile demineralized water. The pH was adjusted to approximately 7.4 with a 5% solution of sodium bicarbonate (usually about 25 ml of bicarbonate solution per liter of diluted Medium 199). The adenosine triphosphate and labile compounds were added just before use of the medium. Penicillin and streptomycin were added to a final concentration of 100 units and 100 µg, respectively. All Medium 199 used for monkey kidney cultures contained 2% calf serum (heated 56°C for 30 min.) for initial growth of cells and 1 or 2% calf serum for maintenance of cells. Packed cells were resuspended in Medium 199 containing 2% calf serum at a ratio of 1 ml of cells to 200 ml of media. This suspension usually contained about 400,000 cells per ml. Tubes were inoculated with 1.0 ml of suspension, 2-ounce bottles with 6 ml, 8-ounce bottles with 20 ml, and 32-ounce

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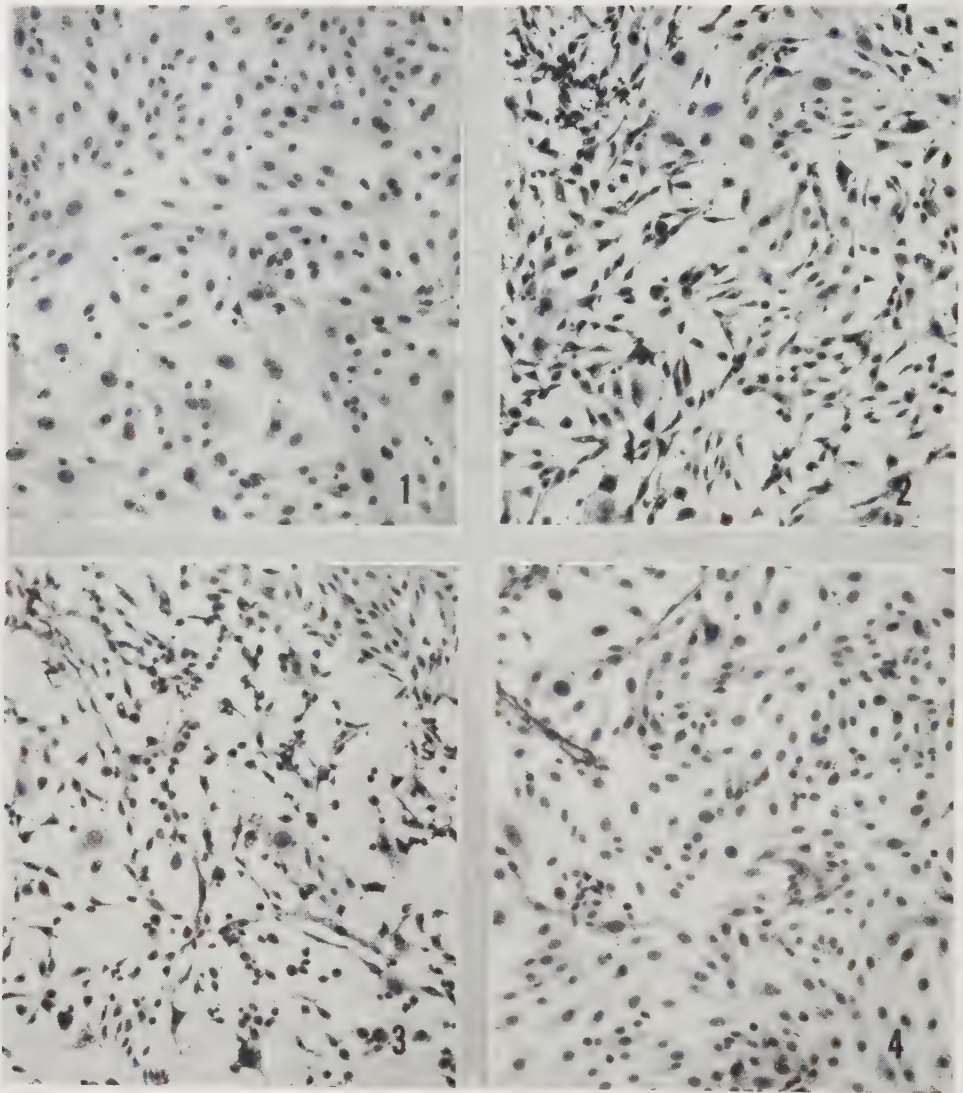


PLATE I. Appearance of refrigerated monkey kidney tissue cultures (Giemsa $\times 80$).

FIG. 1. Monkey kidney cells after 7 days incubation.

FIG. 2. Monkey kidney cells after 7 days incubation and 2 wk refrigeration.

FIG. 3. Monkey kidney cells after 7 days incubation and 4 wk refrigeration.

FIG. 4. Monkey kidney cells after 7 days incubation, 4 wk refrigeration, and 24 hr reincubation.

bottles with 50 ml of suspension. Cultures were closed with rubber stoppers and incubated at 34-35°C. Cells grew to a confluent sheet in 5-7 days.

Results. Storage of monkey kidney tissue cultures. After the cells had grown to a confluent sheet, the cultures were moved to cold storage (4-6°C), using care that the nutrient media covered the cell sheet during storage.

The pH of the medium which covered the cells was acid (clearly yellow to phenol red) when the cultures were transferred from the incubator to cold storage. In some instances the fluid was replaced with fresh Medium 199 before the cultures were refrigerated. At various intervals the cultures were removed from the cold, the fluid replaced with fresh Medium 199 containing 2% calf serum, and the cul-

TABLE I. Sensitivity of Refrigerated Monkey Kidney Cell Cultures to Poliovirus.

| Date cell lot prepared | Time refrigerated | Time incubated after refrigeration | Method of titration | Infective doses/0.5 ml | | |
|------------------------|-------------------|------------------------------------|---------------------|------------------------|------------|------------|
| | | | | Type I | Type II | Type III |
| 2/25/57 | 3 wk | 24 hr | Plaque | $10^{6.7}$ | | |
| 25 | 3 " | 10 days | " | $10^{7.0}$ | | |
| 8/ 1 | 6 " | 48 hr | Roller tube | $10^{6.2}$ | | |
| 9/ 9 | 0 | | " " | $10^{6.5}$ | | |
| 8/ 9 | 3 wk | 24 hr | Plaque | $10^{6.9}$ | $10^{7.3}$ | $10^{6.9}$ |
| 9/ 3 | 0 | | " | $10^{6.7}$ | $10^{7.3}$ | $10^{6.7}$ |
| 8/13 | 26 days | 24 " | Roller tube | $10^{6.4}$ | | |
| 13 | 27 " | | " " | $10^{6.8}$ | | |
| 9/ 9 | 0 | | " " | $10^{6.2}$ | | |
| 8/ 1 | 6 wk | 7 days | Plaque | $10^{6.7}$ | $10^{6.7}$ | $10^{6.4}$ |
| 9/23 | 0 | | " | $10^{6.8}$ | $10^{6.7}$ | $10^{6.7}$ |

tures re-incubated at 34-35°C for 24-48 hours.

The typical appearance of refrigerated cultures is shown in Plate I. It can be seen that as the time of refrigeration increases to 2 weeks (Fig. 2) and to 4 weeks (Fig. 3) the cells pull apart, round up, and leave numerous intercellular spaces. The cells again spread to a full sheet within 24 hours after re-incubation at 34-35°C (Fig. 4).

During the past 6 months over 5,000 cultures from 25 lots were successfully held in the cold for periods up to 6 weeks. Some lots survived longer than others. However, the viability of cultures in any one lot was uniform in that all cultures of a particular lot survived or degenerated within the same time period. In certain instances cultures stored 5-6 weeks required incubation at 34-35°C for periods up to 72 hours for re-sheeting, although in general 24-48 hours were adequate.

Susceptibility of stored cells to poliovirus. Studies[†] of the sensitivity of stored tissue cultures to polioviruses were performed by titrating type I Mahoney,[‡] type II MEF₁, and type III Saukett strains by the plaque(4) method. Other studies were made with the type I Mahoney virus using both the plaque and roller tube methods. In the latter case where only

type I Mahoney reference virus was used, control titrations on non-refrigerated cells were not always included inasmuch as a comparison could be made with the generally accepted titer of $10^{6.5}$ for the N.I.H. reference virus preparation.

Representative results of comparative titrations on refrigerated and non-refrigerated cultures are shown in Table I. It may be seen that refrigerated cultures stored as long as 6 weeks at 4-6°C retain their sensitivity to polioviruses.

A comparison of poliovirus titrations in refrigerated cells (lot prepared on 8/13/57) directly inoculated with poliovirus, and refrigerated cells from the same lot allowed to incubate 24 hours at 34-35°C before being used, is also shown in Table I. The results of this test show no significant difference in the sensitivity of stored cells regardless of whether the cells were allowed to sheet out again before the virus was titered.

Storage of other types of cultures. Storage by refrigeration has been attempted for 3 cell lines; these include HeLa, K.B., and monkey heart. No consistently successful storage procedure beyond one week's duration has been found for any of these lines. Preliminary results indicate that freshly prepared cultures of rabbit kidney will survive at least 2 weeks of cold storage.

In this laboratory, freshly trypsinized monkey kidney tissue held as packed cells at 4-6°C lose viability within 4-5 days with 50% of loss occurring within the first 24 hours. Similarly, minced kidneys have been stored

[†] Virus studies kindly performed by Dr. S. Baron of Division of Biologic Standards.

[‡] The virus preparation used was the N.I.H. reference virus, lot No. 4 which has approximately $10^{6.5}$ TCID₅₀/0.5 ml, and is used as reference virus for production of poliomyelitis vaccine in the United States.

up to 10 days at 4-6°C with recovery of viable cells after trypsinization decreasing sharply after 24-48 hours of cold storage.

Discussion. Since cells held up to 6 weeks at 4-6°C resume growth when returned to the 34-35°C incubator, it is assumed that refrigerated monkey kidney tissue cultures do not "age" significantly during the storage period. Cultures held 3-4 weeks at incubator temperature become very granular whereas cultures stored at 4-6°C for the same interval and then returned to the incubator, have the appearance of young cells and do not show the granularity of age until an additional 3-4 weeks at incubator temperature have elapsed. It is not imperative that the nutrient media be replaced with fresh media before the cultures are refrigerated although it appears that cultures recover more rapidly if the medium is changed before the cells are stored.

The advantages of storing cells are apparent. Cultures not immediately needed can be stored under refrigeration for use at a later date. Similarly, in situations where the supply of monkey kidneys and the need of cultures do not coincide, one can prepare cul-

tures when the tissue is available and hold them in the cold for later use. A bank of tissue cultures can be maintained for immediate service.

Perhaps one of the greatest advantages of using stored cells is that while the majority of a cell lot is refrigerated, an aliquot can be incubated and studied for the presence of simian agents, foamy agents, and non-specific degeneration before the cell lot is used for virus studies, vaccine production, or the safety testing of poliomyelitis vaccine.

Summary. Monkey kidney tissue cultures survive refrigeration at 4-6°C for periods up to 6 weeks. Cultures so treated can be returned to an incubator and maintained for 3-4 weeks at 34-35°C. Refrigerated cultures appear to retain full sensitivity to polioviruses.

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A Confirmatory Test for Mephnesin-Like Action of a Compound on Mice. (23738)

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The central paralyzing action of mephnesin, producing relaxation of the skeletal muscles in the limbs of an animal, is a unique property for agents of this pharmacologic type(1). This property, which causes loss in the ability of a mouse to right itself when placed on its back, has been employed for screening mephnesin-like compounds(2). Mephnesin also causes loss of pinna reflex and corneal reflex in mice; and by observing which reflex is lost first one may distinguish mephnesin from barbiturates, choral hydrate, and other hypnotics. Under the influence of mephnesin, the pinna reflex disappears before the corneal, whereas with other drugs

two reflexes disappear in a reverse order(3). The confirmatory test reported here for the mephnesin-like action of a compound is based on the observation that a minimal paralyzing dose of mephnesin, causing the loss of righting reflex in mice, is ineffective in suppressing the Straub-Hermann mouse tail reaction due to morphine.

Methods. Male albino mice weighing 18-22 g were used. The test compound in solution or suspended in gum acacia was given to 10 animals at each dose level. Subsequently, depressive signs and symptoms, such as quietness, ataxia, loss of grasping reflex, and loss of righting reflex, were noted. Some time

TABLE I. The Influence of Certain CNS Depressants on Straub-Hermann Mouse Tail Reaction to Morphine (20 mg/kg intramusc.).

| Compound | Dosage | | Symptoms* | Tail reaction | | | |
|----------------|-----------------|--------------------------|-----------|-----------------|-------|------|------|
| | mg/kg intraper. | Interval before morphine | | Mean \pm S.E. | Diff. | "T" | "P" |
| Control | | | | 1.53 \pm .10 | | | |
| Mephenesin | 200 | 0 | L.R.R. | 1.47 \pm .17 | .06 | .3 | .1 |
| | 250 | 0 | " | 1.07 \pm .10 | .46 | 2.9 | .01 |
| | 300 | 0 | " | .07† | | | |
| | 350 | 0 | " | .00 | | | |
| Zoxazolamine | 100 | 15 min. | " | 1.33 \pm .12 | .20 | 1.2 | .1 |
| | 250 | 15 | " | .00 | | | |
| Phenobarbital | 50 | 30 | Quiet | 1.18 \pm .15 | .35 | 1.9 | .05 |
| | 100 | 30 | L.G.R. | .23 \pm .09 | 1.30 | 8.6 | <.01 |
| | 150 | 30 | L.R.R. | .00 | | | |
| Barbital | 100 | 30 | Quiet | 1.03 \pm .14 | .50 | 3.3 | .01 |
| | 150 | 30 | L.G.R. | .44 \pm .12 | 1.09 | 6.4 | <.01 |
| | 200 | 30 | L.R.R. | .00 | | | |
| Chloralhydrate | 100 | 15 | Quiet | 1.06 \pm .06 | .47 | 3.8 | .01 |
| | 150 | 15 | " | .82 \pm .09 | .71 | 5.0 | <.01 |
| | 250 | 15 | L.R.R. | .21 \pm .07 | 1.32 | 10.3 | " |
| | 300 | 15 | " | .00 | | | |
| Meprobamate | 150 | 15 | Quiet | .91 \pm .22 | .62 | 2.8 | .01 |
| | 200 | 15 | L.R.R. | .00 | | | |
| Dilantin | 50 | 3 hr | Quiet | .97 \pm .14 | .56 | 3.2 | " |
| | 100 | 3 | L.G.R. | 1.05 \pm .12 | .48 | 2.8 | " |
| | 200 | 3 | L.R.R. | .55 \pm .04 | .98 | 8.4 | <.01 |

* L.G.R. = Loss of grasping reflex. L.R.R. = Loss of righting reflex.

† 4/10 mice showed a slight reaction toward end of one hr.

later, depending upon the duration of effect of a test compound determined previously, a challenging dose of morphine sulfate (20 mg/kg) was then injected intramuscularly. Fifteen minutes after morphine administration, the mouse tail reaction as well as the depressive symptoms were observed and recorded at 5-minute intervals for a period of 50 minutes. The numerical ratings for tail erection are those adopted by Juul(4) as follows: 1 = 45°, 2 = 90°, 3 = 180° from the table plane; 1.5 and 2.5 = intermediate reactions between 45°-90° and 90°-180°, respectively. The mean and standard errors of tail response for 10 mice are computed from the averages of 10 readings for each animal(5).

Results. As shown by the data in Table I, loss of righting reflex in mice resulted following intraperitoneal injection of 200 mg/kg of mephenesin or 100 mg/kg of zoxazolamine. At these levels, however, they did not significantly affect the mouse tail reaction to morphine. Only at higher paralytic doses was the tail response to morphine suppressed by these drugs. In contrast the morphine-in-

duced tail reaction in mice was markedly impaired under the influence of phenobarbital, barbital, chloral hydrate, and meprobamate at sedative dose-levels and completely prevented by them at minimal paralytic dosages. A suppressive effect of Dilantin on the tail reaction was obtained also at non-paralytic doses, although the inhibition of the tail reaction was not complete with this drug even at a minimal lethal dose of 200 mg/kg. Therefore, it is possible, by the mouse tail reaction to morphine and depressive symptoms at appropriate dosages, to distinguish clearly a depressant property of mephenesin from that of Dilantin or a barbiturate-like compound.

The difference in their depressant effect at threshold and at higher paralyzing doses upon the morphine-induced tail reaction in mice indicates evidently the 2 sites or mode of actions of mephenesin or zoxazolamine on the central nervous system. It is commonly held that these drugs apparently affect the CNS structures in an ascending order. The spinal cord may be the only site to be affected by the mephenesin type of drug at low dosages.

This test, therefore, may be utilized also to determine the dosages of these compounds for their depressant effect on different parts of the central nervous system. Since high paralyzing doses of mephenesin or zoxazolamine are required to antagonize the stimulating action of morphine, it appears to support the opinion of some that higher centers above the spinal cord are involved in the mouse tail reaction to morphine(6,7).

Summary. The mouse tail reaction to morphine is not suppressed by mephenesin or zoxazolamine at minimal paralyzing dose levels. On the other hand, it is markedly suppressed by phenobarbital, chloral hydrate, meprobamate, and Dilantin at sedative dosages. The lack of a suppressive influence of an agent on the morphine-induced tail re-

sponse in mice at paralyzing doses is suggested as a confirmatory test for mephenesin-like property.

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Metabolic Fate of S³⁵ Administered to Rabbits as Sulfate.* (23739)

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In recent years studies making use of radio-sulfur (S³⁵) have demonstrated that inorganic sulfur can be used by ruminants for the synthesis of cystine and methionine(1,2,3). These studies suggest that the extensive microbial synthesis occurring in the rumen may be responsible for the appreciable extent to which inorganic sulfur is utilized in the formation of organic sulfur compounds. Several recent studies have reported the pattern of tissue uptake of S³⁵ observed in rabbits following administration of S³⁵-labeled sulfate (4,5,6). In the case of the last of the above cited studies, it was observed that the collaring of rabbits in such a manner as to prevent coprophagy, which apparently is habitually practiced by rabbits in the absence of a

restraining device, results in an appreciable lowering of the uptake of S³⁵ in the blood, liver, spleen, muscle and intestinal tissues. The present study was carried out to determine if any conversion to organic form could be detected after S³⁵-labeled sulfate was administered to rabbits.

Procedure. A dose of approximately 0.9 millicurie of S³⁵ in the form of sodium sulfate plus 0.22 mg of carrier sodium sulfate was given by stomach tube to each of 4 littermate, female New Zealand white rabbits. One pair of rabbits was dosed at 10 weeks of age, and the others at 12 and 14 weeks of age. The animals were fed a commercial pelleted ration containing 0.23% total sulfur and 16.9% crude protein. The methionine content was 0.22% and there was 0.18% cystine present. During the collection period 2 of the rabbits wore plywood collars to prevent coprophagy. The collars were made of 1/4-inch thick plywood. They were fitted closely about the neck by means of a rubber cushion which lined the neck opening, and had outside diameters of about 10 inches. The ex-

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TABLE I. Summary of S³⁵ Excretion and Tissue Uptake Data.

| | Uncollared rabbits | Collared rabbits |
|--|--------------------|------------------|
| S ³⁵ recovered in urine, % of dose | 46.9 | 49.5 |
| S ³⁵ recovered in hard feces, % of dose | 12.1 | 20.3 |
| S ³⁵ recovered in soft feces, % of dose | 0 | 17.9 |
| Ear cartilage S ³⁵ , % dose/g | .0320 | .0265 |
| Gastrocnemius muscle S ³⁵ , % dose/g | .0008 | .0003 |
| Kidney S ³⁵ , % dose/g | .0056 | .0024 |
| Clipped skin S ³⁵ , % dose/g | .0072 | .0034 |
| Liver S ³⁵ , % dose/g | .0044 | .0008 |
| % liver S ³⁵ in sulfate form | 29.3 | 86.1 |
| <i>Idem</i> methionine form | 53.7 | 10.6 |
| " cystine form | 17.0 | 3.3 |

creta were collected and blood samples were obtained at various intervals during the 4 days following dosing, and then the animals were sacrificed. Selected tissues were analyzed for total S³⁵ concentration by use of a technique which has previously been described(6). Samples of liver weighing about 3 g from one collared and one uncollared rabbit were hydrolyzed in individual sealed tubes for 8 hours with 25 ml of 6 *N* hydrochloric acid at a temperature of 115°C. The excess acid of the resulting hydrolysates was evaporated under partial vacuum at a temperature below 35°C. The residue was taken up in distilled water and an aliquot was applied to a 0.9 x 100 cm Dowex-50 ion-exchange column in the hydrogen cycle. The S³⁵ of the samples was fractionated by elution of the column with hydrochloric acid by the method of Stein and Moore(7).

Results. During the 4-day period which followed dosing, the uncollared rabbits consumed an average of 104 g of feed per day and gained 114 g in weight. The collared rabbits consumed an average of 127 g of feed per day and lost 77 g in body weight during the 4 days following dosing.

The data for S³⁵ excretion during the 4-day collection period and tissue S³⁵ concentrations at sacrifice are summarized in Table I. Much of the S³⁵ was rapidly absorbed and excreted by the renal pathway. Within 3 hours one of the rabbits of the collared group had excreted

11% of the dose in the urine. Fecal excretion of S³⁵ also was rather rapid. One of the collared rabbits excreted 0.4% of the dose in the soft feces within 3 hours of dosing. The soft feces are the type that are normally ingested by an unrestrained rabbit. The appearance of S³⁵ in the feces with such rapidity suggests that part of the dose was rapidly absorbed and then excreted into the lower part of the intestinal tract.

The values obtained for blood S³⁵ concentration at various time intervals (Fig. 1) give an indication of the speed with which the S³⁵ was absorbed. The peak concentration appears to have occurred around 6 hours after dosing. Blood S³⁵ concentrations were appreciably lower in the case of the collared rabbits than those observed in the case of the uncollared rabbits from the sample taken 3 hours after dosing until the time of sacrifice. At 12, 24, and 48 hours after dosing, the blood S³⁵ concentrations of the collared rabbits were about one-half as high as those of the uncollared rabbits, and at 72 and 96 hours after dosing they were less than one-half as high.

The tissue total S³⁵ concentration values (Table I) confirm the results of a previous study on the effect of coprophagy on labeled S³⁵ uptake by rabbits(6). While the ear cartilage values did not differ widely, there were considerably higher S³⁵ concentrations in the liver, kidney, clipped skin and gastrocnemius

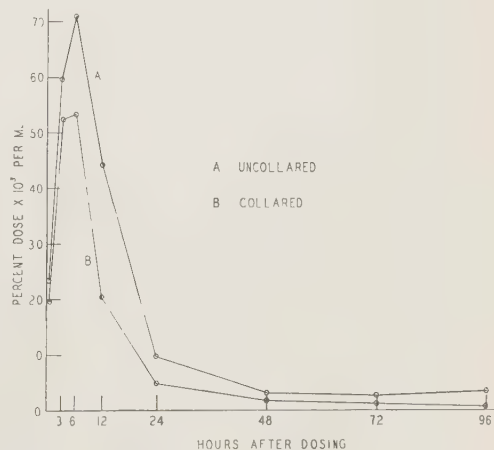


FIG. 1. Radiosulfur concentration in blood of rabbits at various intervals after oral administration of labeled sulfate. Points represent averages of values for the 2 rabbits of each group.

muscle of the rabbits that were not collared than occurred in collared animals. The results of the fractionation of the S^{35} in liver hydrolysates (Table I) indicate that there was a significant amount of S^{35} incorporated into cystine and methionine in the case of the uncollared rabbit, and that a marked reduction in the amount of S^{35} appearing in organic forms in the collared rabbit. There was about 30 times as much S^{35} -labeled cystine and methionine in the liver of the uncollared rabbit, and the S^{35} -labeled sulfate fraction was twice as great as was the case in the liver of the collared rabbit. These results suggest that the increased amounts of labeled cystine and methionine found in the liver of the uncollared rabbit may have been acquired as the result of fecal refection, followed by the digestion and assimilation of organic sulfur compounds synthesized by intestinal microorganisms.

Summary. The metabolic fate of S^{35} -labeled sulfate in young rabbits was studied. Absorption of S^{35} after labeled sulfate was administered by stomach tube was very rapid,

with the peak appearing in blood around 6 hours after dosing. Some synthesis of labeled cystine and methionine took place, with S^{35} -labeled cystine and methionine appearing in the liver. When the habitual practice of coprophagy was interfered with by fitting the rabbit with a collar which prevented fecal refection, the amount of labeled cystine and methionine was greatly reduced.

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Synaptic Effects of Systemic γ -Amino Butyric Acid in Cortical Regions of Increased Vascular Permeability. (23740)

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(Introduced by Harry Grundfest)

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Topically applied to the cerebral cortex, aliphatic ω -amino acids exert rapid, reversible synaptic effects dependent on carbon chain length(1-3). Electrophysiological analyses indicate that the acids with less than 5 carbons selectively block excitatory synapses on apical dendrites, whereas longer chained ω -amino acids selectively block inhibitory

synaptic electrogenesis. Of the group with less than 5 carbons, γ -amino butyric acid (GABA), the most potent inactivator of dendritic excitatory synapses, occurs in relatively high concentration in normal brain(4,5). Selective blockade of excitatory dendritic synapses following topical application of GABA has been shown to account for the alterations induced in a variety of evoked cortical responses(3). In contrast to the effects caused by topical application, intravenous administration of GABA produces neither central synaptic actions nor detectable increase in brain concentration of the amino acid(6). These results suggest that under

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ordinary circumstances GABA does not effectively penetrate the blood-brain barrier; its behaviour in this respect being similar to that of one of its precursors, glutamic acid (7). Rapid penetration of GABA into the brain following alterations in the permeability characteristics of the barrier, should therefore produce synaptic effects similar to those resulting from topical application. Although little is known about the essential nature of the barrier, a variety of agents, chemical and physical, are capable of affecting it. Two of these, ethyl chloride and chloroform-methanol permit local destruction of the barrier with preservation of physiological activity in most regions of barrier loss. Under these conditions systemic administration of ω -amino acids, exerts synaptic actions in regions of local barrier breakdown comparable to those produced by topical application. The present report primarily concerns the overt effects of systemically administered GABA.

Methods. Twenty succinylcholine-paralyzed cats were prepared under ether anesthesia and with procaine infiltration of the skin and muscles at the operated sites for cortical registration of evoked dendritic postsynaptic potentials in a manner similar to that previously described in detail(8). In some of these, small amounts of pentobarbital sodium (15 mg/K) were administered prior to recording periods. Local breakdown of the blood-brain barrier was produced either by application of a few drops of chloroform-methanol (2:1) solution to the cortical surface, or by restricted freezing with ethyl-chloride utilizing the technique of Morrell and Florenz(9) for producing experimental epileptogenic lesions. Cortical regions of barrier loss were delineated by vital staining with Evans blue dye (W761-1)[†] injected prior to, or immediately after, production of the lesion. Spontaneous or evoked potentials recorded in these regions over long periods of observation exhibited no change attributable to the presence of the dye in the cortex. Although a detailed analysis of the histological and electrophysiological changes occurring in cortex

treated with these agents will be presented elsewhere(10), in the experiments described below the criteria for viability were evaluated only in physiological terms. It should be noted, however, that application of ethyl-chloride or chloroform-methanol resulted in localized destruction of cytoarchitecture (Fig. 1), more pronounced following ethyl chloride freezing than application of the chloroform-methanol. In all cases staining of a wedge shaped region of the entire thickness of cortex could be readily observed, extending in many instances deeply into the subcortical white matter (Fig. 1A). Of considerable importance was the fact that blood-brain barrier destruction, as evidenced by the penetration of Evans blue, extended well beyond the regions of maximal histological changes. With the exception of the latter regions, of little or no responsiveness, potentials could be readily evoked by surface stimulation in surrounding areas exhibiting marked blood-brain barrier destruction. *Smaller areas of unresponsiveness* to direct stimulation were generally encountered in chloroform-methanol treated regions than in those sprayed with ethyl-chloride. Despite relatively less cellular damage induced by chloroform-methanol, the severity and extent of blood-brain barrier loss produced by this lipid solvent exceeded that caused by ethyl-chloride. *Significant physiological differences* were observed in regions of ethyl-chloride freezing and chloroform-methanol application, related in part, to the degree of localized cellular destruction. Regions treated with chloroform-methanol never gave rise to spontaneous paroxysmal discharges within the period of observation (6-8 hours), while these were repeatedly observed following ethyl-chloride spraying, depending on the cortical location of the lesion. Their presence in regions of barrier destruction permitted analysis of systemically administered amino acids on the activity of the epileptogenic focus. At the conclusion of all procedures, the animals were anesthetized with 30 mg/K pentobarbital sodium and the brain perfused with 10% formalin.

Results. *A. Effects of systemic GABA on evoked dendritic postsynaptic potentials.*

[†] Generously supplied by Warner-Chilcott Laboratories.

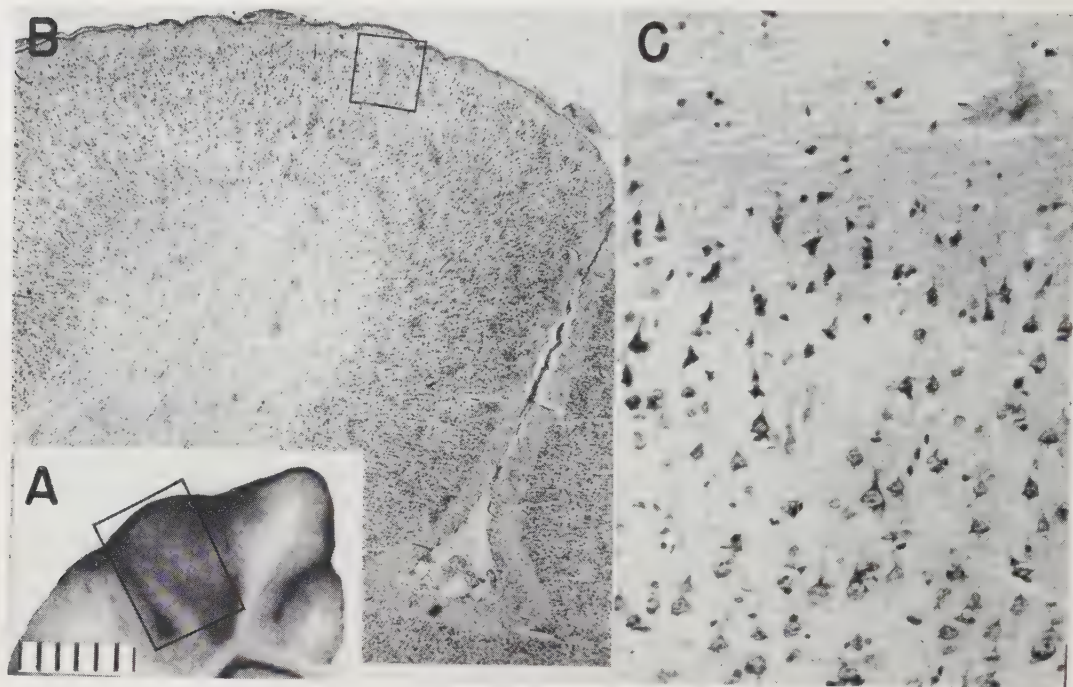


FIG. 1. *A*. Gross appearance of vitally stained fixed brain following application of 3 drops of chloroform-methanol solution to suprasylvian gyrus, 6 hr prior to fixation. Evans blue dye detectable throughout gyrus and deeply into white matter. Evidence of massive blood-brain barrier destruction also observed in adjacent gyri. Scale in mm. *B*. Nissl preparation, of rectangular area demarcated in *A* magnified ten-fold, showing regions of normal and abnormal cytoarchitecture. In this preparation absence of responsiveness to direct stimulation was limited to a small region of maximum destruction. *C*. Ten-fold enlargement of region in *B*, taken through area of maximal cellular destruction.

Topical application of GABA reverses the surface negative evoked potential of the cerebral cortex to positivity by "unmasking" counter-vailing hyperpolarizing postsynaptic potentials, when the depolarizing responses are blocked (1). Responses evoked from wide areas of blood-brain barrier destruction, were altered in a similar way by systemic amino acids (Fig. 2), whereas no effects were detectable in unstained cortical regions of normal vascular permeability.

In stained cortex following an ethyl chloride lesion, the evoked response (Fig. 2*A*, upper channel recording) was essentially similar to that recorded from a contralateral, homologous site (Fig. 2*A*, lower channel). Within 15 seconds after intravenous administration of 50 mg/K GABA, rapid abolition of evoked surface negativity occurred in stained cortex (shown in single channel records, *B*) without changes in responses from unstained cortex (*C*). Blockade of excitatory

p.s.p.'s and unmasking of dendritic inhibitory p.s.p.'s in stained regions (*B*) persisted for more than 45 minutes, gradually reversing and recovering (*D*) at the end of 1 hour. Similar results were encountered in minimally narcotized preparations (*I-L*).

In chloroform-methanol treated cortex blockade of excitatory synapses on apical dendrites also occurred with dramatic onset following intravenous GABA (Fig. 2, *E-H*). Effects were detectable (lower channel) with as little as 8 mg/K (*F*), complete blockade being produced by an additional 25 mg/K (*G*). Recovery was considerably delayed in these preparations, for as long as 90 minutes (*H*).

The depth of the blood-brain barrier lesions produced in sensorimotor cortex by application of chloroform-methanol (Fig. 1) permitted testing the effects of systemic GABA on axo-somatic synaptic activity generated by collateral excitation of cortico-spinal neurons

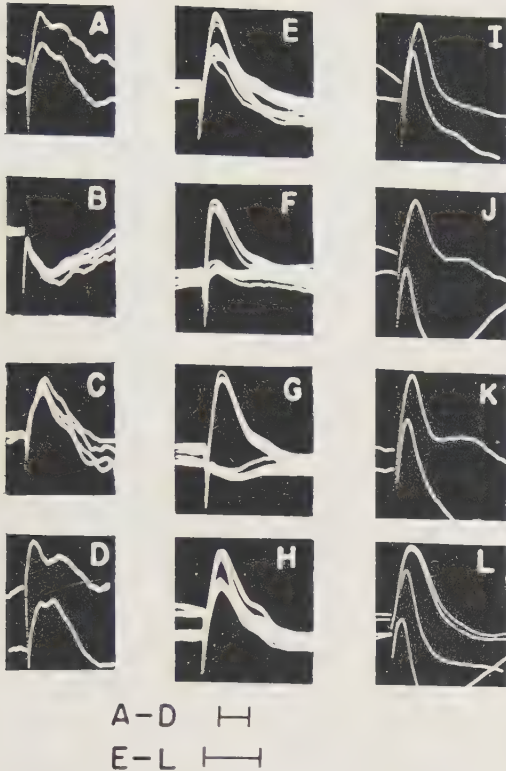


FIG. 2. Differential action of systemically administered γ -amino butyric acid (GABA) in cortical regions of blood-brain barrier loss. In all records dendritic postsynaptic potentials recorded monopolarly following direct cortical stimulation. In this and subsequent records negativity at stigmatic electrode indicated by upward deflection. *A-D*: (unanesthetized-paralyzed cat, ethyl-chloride induced barrier lesion). Upper channel records in *A* and *D* and *B* from stained cortex; lower channel records and *C*, normal cortex. *B* and *C* superimposed responses evoked 40-50 sec. after GABA, 50 mg/kg intrav. *D*, 60 min. later. *E-H*: (pentobarbitalized cat, 15 mg/kg; chloroform-methanol induced barrier lesion). Upper channel records from normal cortex, lower channel records from regions of barrier breakdown. In *E*, *F*, after 8 and 25 mg/kg GABA, respectively. *H*, 90 min. after *G*. *I-K*: (ethyl-chloride induced barrier lesion). *I*, dendritic p.s.p.'s before inj. of 25 mg/kg GABA. Effect of inj. detectable only in area of blood-brain barrier loss (lower channel records) is maximal at 1 min. (*J*) and slowly reversible (45 min., *K*). *L*, superimposed records before and after another similar inj. Time calibrations 20 msec.

or by indirect stimulation of specific thalamo-cortical afferents. Stimulation of the cortical surface in such regions evoked discharges into the pyramidal tract consisting of directly and indirectly relayed components, the latter presumably being axo-somatically generated

(2,8). Comparison with axo-dendritic activity produced by direct surface stimulation in stained cortical regions and with responses evoked by indirect stimulation of thalamo-cortical fibers from the cortical surface (Fig. 3), indicates that, like the topically applied, the systemically administered GABA (30 mg/K) is without effect on axo-somatic synaptic activity. Abolition of surface negative dendritic components, however, unmasks surface positivity (Fig. 3A2, B2).

B. Effects of γ -amino butyric acid on experimental epileptogenic focus. Cortical regions treated with ethyl-chloride often exhibited spontaneous paroxysmal activity(9). High-voltage discharges of varying frequency

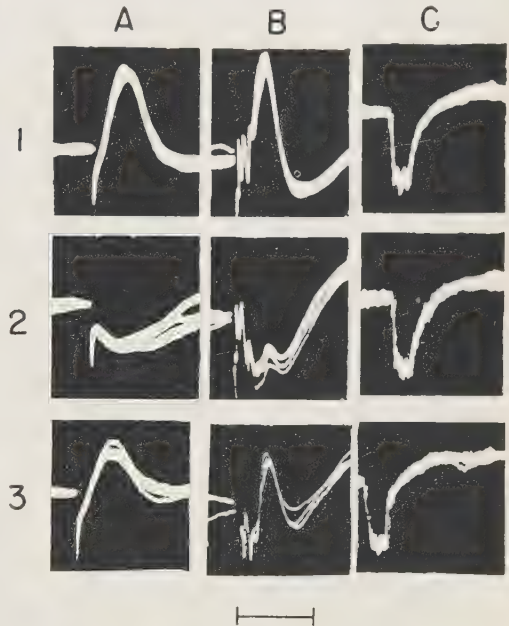


FIG. 3. *A*: Dendritic p.s.p.'s evoked by surface stimulation, post-sigmoid gyrus. *B*: Responses evoked from same site as in *A*, following strong stimulation of pre-cruciate cortex which also evokes discharges in the cortico-spinal tract, recorded at the mid-olivary level in the medulla (*C*). Entire anterior and post-sigmoid gyri treated with chloroform-methanol solution 2 hr prior to recordings. *1*, controls; *2*, effects induced 1 min. after 30 mg/kg GABA, intrav.; *3*, beginning recovery, 60 min. after *2*. Note unmasking of surface positive dendritic response (*A2*, *B2*) with no effect on axo-somatically generated activity (*C2*). Alterations in spike sequence in *B2* presumably due to developing slow surface positivity. Individual spike-like responses signaling axo-somatic discharges are not abolished by the systemically administered amino acid. Time 20 msec.

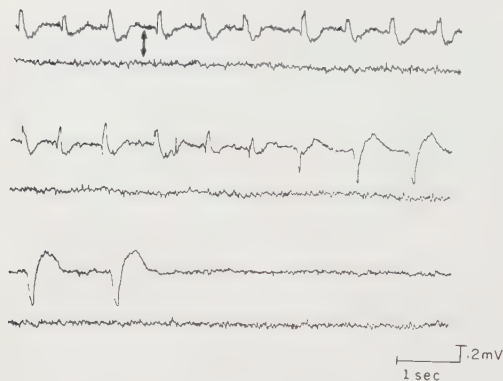


FIG. 4. Effects of systemic GABA on paroxysmal discharges spontaneously developing in region of blood-brain barrier loss induced by ethyl-chloride. Continuous record. *Upper channel*, monopolar recording from center of spike focus, post-sigmoid gyrus. *Lower channel*, bipolar recording from normal cortex, ant. suprasylvian gyrus. Focus discharged regularly for 2 hr prior to start of inj. (signaled by arrow and continued throughout recording). Total inj. was 30 mg/kg, but less than 15 mg/kg was sufficient to induce prompt reduction and reversal of spontaneous paroxysmal discharge prior to its disappearance.

were always localized well within areas of intense cortical staining with Evans blue dye and consequently were available to analysis following intravenous administration of GABA. Regularly discharging foci of relatively low frequency were affected by GABA in low concentration (10-15 mg/K). Monopolarly recorded negative-positive discharges were reduced, *reversed* and abolished for periods depending on the quantity of injected GABA. Injections of 30 mg/K (Fig. 4) abolished paroxysmal discharges for hours, none reappearing for the remainder of the experimental period in many cases. In preparations with rapidly discharging foci (3-5/sec. or more) GABA was less effective. In these, abolition of paroxysmal activity could be effected for short periods (5-10 min.) with 50-75 mg/K GABA. Discharges recurring after this were always at a lower frequency than prior to injection. Abolition of paroxysmal discharges was not accompanied by significant alteration in background electrocortical activity.

Comments. The foregoing results indicate that the powerful blocking action of γ -amino butyric acid on depolarizing synapses of apical dendrites is independent of its mode

of administration. However, in contrast to the transient effects produced by topical application, prolonged, slowly reversible blockade of excitatory dendritic synapses occurs in regions of blood-brain barrier loss, following intravenous injection of GABA. As a consequence of this, paroxysmal discharges spontaneously developing in ethyl-chloride treated cortical regions are abolished or markedly reduced in frequency for long periods of time. The synaptic mechanisms responsible for polarity reversal of paroxysmal discharges prior to their abolition are not revealed by the present experiments. Topical application of GABA induces similar reversals in such discharges, but only rarely abolishes them, suggesting that systemic administration delivers more effective concentrations at excitatory axo-dendritic synaptic sites than is achieved by topical application.

Failure to demonstrate significant alterations in spontaneous or evoked activity in normal cortex following intravenous administration of GABA is therefore directly attributable to the presence of the blood-brain barrier. The latter may also be an effective mechanism for preventing diffusion of free GABA from the brain into the blood stream. In local cortical regions of increased vascular permeability the marked rapid electrophysiological alterations shown above are consistent with rapid increases in GABA content. Conversely, outflux of endogenously synthesized GABA from brain in such regions may also result in equally profound functional disturbances. Thus, in the presence of increased blood-brain barrier permeability, relatively small changes in brain concentration of synaptically active free amino acids like GABA may contribute to the production of neurological and psychiatric defects associated with abnormal plasma amino-acid levels such as are commonly observed in various states of hepatic insufficiency (10.)

Finally, the fact that GABA fails to block excitatory axo-somatic synapses when delivered to the latter systemically, supports the hypothesis (1-3) that the amino acid acts selectively on axo-dendritic synapses.

Conclusion. Under ordinary circumstances systemically administered γ -amino butyric

acid does not produce electrophysiological effects. Following destruction of the blood-brain barrier the synaptic actions are similar to those produced by topical application. In regions of increased barrier-permeability, intravenous GABA abolishes or reduces the frequency of spontaneous paroxysmal discharges arising from such areas. Therefore systemically administered GABA produces no synaptic effects because of failure to penetrate the blood-brain barrier. By permitting rapid exchanges of GABA between blood and brain during electrophysiological studies following barrier lesions, information was obtained relating to the role of γ -amino butyric acid in the central nervous system not attainable by the method of topical application. Production of experimental, localized regions of blood-brain barrier loss may have applications for the study of substances that are otherwise

denied access to the cells of the central nervous system.

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Calory and Protein Intake as Related to Growth. (23741)

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Numerous recent papers on calory and protein nutrition of fowls have been concerned with the "Calory-Protein Ratio." This ratio is the nutritional caloric value of the diet divided by the percentage of protein. There has appeared no consistent relation of this ratio to biological results unless either protein or caloric value of the diet has been relatively constant.

Exp. In an experiment on protein and caloric needs, B. B. Bronze hens on range were provided definite intakes of practical formula feeds and grain per day (Table I). Protein intake was calculated from analyses. Caloric intake was expressed as "Productive Energy Values" of Fraps, revised by Titus(1). Most of the reports referred to have also employed such values. These have been expressed as calories per lb. of feedstuff. Correlation of calory-protein ratio to growth results was found to be negligible. It was observed that the *product* of the caloric and

protein intakes showed a good correlation with body weights (coefficient 0.83).

Discussion. The ratio remains the same whether calculated from composition of diet or from intakes, and is independent of the magnitude of intakes. Hence such anomalous findings appear as a decrease in growth when the ratio is increased, due to decreased total feed or decreased protein intake or both(2,3). Protein is represented in the numerator of the ratio as protein calories and in the denominator as percent protein. This implies that all protein simultaneously is consumed to produce calories, and also is utilized for growth purposes, which, obviously, is impossible. Since the ratio includes all the protein calculated both as calories and as percentages of protein the ratio may be expressed:

$$\text{Calory-protein ratio} = \frac{\text{Protein calories}}{\text{Percent protein}} + \frac{\text{Non-protein calories}}{\text{Percent protein}}$$

TABLE I. Protein and Caloric Intakes of B. B. Bronze Turkey Hens in Relation to Growth.

| Group No.* | Total calories/hen/day† | Protein/hen /day, g | Avg wt at 26 wk, kg‡ | Calory-protein ratio | Non-protein calory-protein product |
|------------|-------------------------|---------------------|----------------------|----------------------|------------------------------------|
| 1 | 450 | 39.5 | 7.02 | 51.7 | 29.7 |
| 2 | 429 | 40.9 | 7.24 | 47.7 | 28.5 |
| 3 | 378 | 39.5 | 6.73 | 43.8 | 23.4 |
| 4 | 449 | 43.6 | 7.36 | 46.7 | 31.6 |
| 5 | 413 | 43.6 | 7.11 | 43.0 | 28.1 |
| 6 | 377 | 43.6 | 6.87 | 39.2 | 24.7 |
| 7 | 411 | 44.0 | 7.18 | 42.4 | 28.1 |
| 8 | 413 | 38.6 | 6.95 | 48.6 | 26.1 |
| 9 | 416 | 36.8 | 6.64 | 51.3 | 25.5 |
| 10 | 448 | 37.2 | 7.02 | 53.3 | 28.8 |
| 11 | 445 | 40.4 | 7.24 | 50.0 | 29.7 |
| 12 | 537 | 41.8 | 7.70 | 58.4 | 38.8 |

* Approx. 180 hens started/group.

† Productive energy (Fraps) revised by Titus(1).

‡ Exp. started at 18 wk of age with pens of equivalent avg wt.

The first term is a constant. The variability resides entirely in the ratio of non-protein calories to percentage protein. A change in percentage protein involves a change in the numerator of the second term by affecting the percentage of non-protein and an opposite change in the denominator, thereby, causing a large change in the ratio. A wide series of diets containing adequate protein, or more, can have a wide series of ratios and yet yield equivalent biological results, because the protein used for caloric production is nearly equivalent to the carbohydrate displaced by protein. When the products are employed the loss of protein to produce energy is approximately balanced by the gain in calories.

To avoid the ambiguous use of protein an attempt was made to remove the protein calories from the productive energy, leaving only the total non-protein calories. This may amount to an over correction, in variable degree, since some protein is always converted to energy.

From productive energy values for blood meal, fish meal, meat scrap, casein, fat and representative analyses the following productive energy values for the protein component were calculated.

| | Productive energy of protein (cal/lb) |
|------------|---------------------------------------|
| Blood meal | 1270 |
| Casein | 1300 |
| Fish meal | 1240 |
| Meat scrap | 1200 |

Sucrose is listed at 1334 calories and it is

stated that protein has .914 the energy of carbohydrate(1). This leads to a value of 1220 calories. An average of all 5 values was taken at 1250 (2750 cal./kg).

Since quality of protein of a well-balanced diet is indistinguishable as to origin, because of mutual supplementary actions, a single factor may be used to express the caloric equivalent of such protein of a good mixed diet. Using the 2750 factor the total non-protein caloric intake of the turkey hens was calculated. The ratio of non-protein calory intake to protein intake showed a very low correlation with body weights. On the other hand, the non-protein calory intake was multiplied by the protein intake and correlation of the product with hen weights was obtained as a coefficient of 0.92, representing an improvement over the 0.83 first obtained. Such improvement indicates that a more correct manner of relating the data has been employed.

The above observations were applied to other recent data embodying a range of variation of both protein and caloric intakes. For example, data provided by Ferguson *et al.*(2) were calculated to the basis of protein and caloric intake in cases of the most complete diets fed to B. B. Bronze poults. Correlation of calory-protein product and growth was 0.70 and was improved to 0.87 when the caloric term was corrected to non-protein calories. Data of Donaldson *et al.*(3) for young chicks show a correlation coefficient of growth and calory-protein product of 0.74, which was improved to 0.94 when corrected to a non-pro-

tein caloric basis. Sunde(4) has provided data from chick tests which yield little or no correlation of growth with calory-protein ratio, a low correlation with calory-protein product and a good correlation, of about 0.8, of growth with the non-protein calory-protein product. Data with rats have been provided by Sibbald *et al.*(5). While the weights reported are averages of initial and final weights, the growth results are highly correlated with the product of digestible nonprotein calories and digestible protein intakes, (coefficient 0.92). Other examples have been studied with similar results. The relations discussed

above no longer apply when the genetic or physiologic capacities of the animal have been exceeded.

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Comparative Behavior of 16 ECHO Virus Types in Fibroblast-like and Epithelial-Like Human Cell Strains.* (23742)

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One of the characteristics of the ECHO viruses as a group is their ability to proliferate in tissue cultures of rhesus (*Macaca mulatta*) monkey kidney cells(1). Neither cultured kidney cells from other species of monkeys, nor cultured strains of human cells have appeared to be uniformly susceptible to all of the ECHO viruses(1,2,3). This communication reports a systematic study of the ability of 16 ECHO virus types to proliferate in human cell strains of different morphology as well as from different malignant and non-malignant sources.

Materials and methods. Cell strains. One fibroblast-like (Fb-L) and 6 epithelial-like (Ep-L) human cell strains have been investigated. Four of the Ep-L strains have been previously described(4). Of these, Detroit-30A was derived from carcinomatous ascitic fluid, Detroit-32 from bone marrow of a patient with carcinoma, Detroit-98 from normal bone marrow, and Detroit-116P from lymphomatous pleural fluid. Although some of the ECHO viruses appear to proliferate in HeLa cells(1), especially after adaptation(3), the

HeLa strain was included in this study for purposes of comparison. The 2 additional cell strains, Detroit-196 Fb-L and Detroit-196 Ep-L were established as follows. A skin biopsy from a patient with urticaria pigmentosa was minced, washed with basal medium, Eagle (5) (BME), and incubated in 0.25% trypsin solution (Difco, 1:250). The resulting cell suspension was again washed and approximately 10^5 cells were suspended in 8 ml of growth medium (BME 80%, human serum 20%) and deposited into a 3-ounce prescription bottle. After incubation at 36°C for 40 days, fibroblast-like cells were distinguishable among the cellular debris and within the next 10 days a network of Fb-L cells covered the wall of the bottle. These were subcultured according to procedures previously described (4). An inoculum of 4×10^5 cells in 3-ounce bottles yielded averages of 2×10^6 cells in 8 days. After the Detroit-196 Fb-L cells had undergone 6 serial subcultures over a period of 2½ months, islands of polygonal cells appeared in several bottles of the 6th passage. These polygonal cells outgrew the fibroblasts and thus a substrain of epithelial-like cells emerged which has been continuously main-

* Supported by Grants from N.I.H.

TABLE I. Histories of Human Cell Strains.

| Cell strain | Tissue source | Diagnosis of patient's disease | Biopsy material containing malignant cells | Life of strain* (mo) |
|------------------|----------------------------------|--|--|----------------------|
| Detroit-196 Fb-L | Skin | Urticaria pigmentosa | None | 13 |
| -196 Ep-L | (Derived from 196 Fb-L cultures) | " " | " | 10 |
| - 32 " | Sternal marrow | Carcinoma, primary site undetermined | Pleural fluid | 30 |
| - 98 " | " " | Refractive error; no malignancy detected | None | 26 |
| - 30A " | Ascitic fluid | Carcinoma of the breast | Mass in breast; ascitic fluid | 33 |
| -116P " | Pleural fluid | Lymphosarcoma | Pleural fluid | 25 |

* To Nov. 1, 1957.

TABLE II. Behavior of 16 ECHO Viruses in Cultures of Detroit-196 Fb-L and -196 Ep-L Strains of Human Cells.

| ECHO virus type | TCD ₅₀ * in M.K. cultures | Dilution of inoculum | Passage in Detroit-196 Fb-L | | | Passage in Detroit-196 Ep-L | | |
|-----------------|--------------------------------------|----------------------|-----------------------------|---------|-------------------------|-----------------------------|-----|-------------------------|
| | | | 1st CPE | 2nd CPE | 3rd TCD ₅₀ † | 1st CPE‡ | 2nd | 3rd TCD ₅₀ ‡ |
| 1 | 7.2 | 1:100 | + | + | 5.8 | + | § | — |
| 2 | 6.8 | " | + | + | 4.6 | + | | — |
| 3 | 6.2 | " | + | + | 4.6 | + | | — |
| 4 | 7.5 | " | + | + | 5.5 | + | | — |
| 5 | 7.5 | " | + | + | 5.7 | + | | — |
| 6 | 6.8 | " | + | + | 5.0 | + | | 3.2 |
| 7 | 6.6 | " | + | + | 6.4 | + | | — |
| 8 | 5.6 | | + | + | 6.5 | + | | — |
| 9 | 4.5 | 1:100 | + | + | 4.5 | — | | — |
| 10 | 4.5 | " | — | | | + | | 2.5 |
| 11 | 5.7 | " | + | + | 4.5 | + | | — |
| 12 | 6.5 | " | + | + | 4.4 | — | | — |
| 13 | 6.4 | | + | + | 5.5 | + | | — |
| 14 | 7.3 | 1:100 | + | + | 5.4 | + | | — |
| 15 | 1.5 | Undiluted | + | + | 3.2 | — | | — |
| 16 | 4.5 | 1:100 | + | + | 4.8 | — | | — |

* Expressed as log dilution/0.1 ml, based on cytopathogenic effect (CPE).

† As assayed in monkey kidney cultures.

‡ Presence of virus was determined by CPE in monkey kidney cultures.

§ Blind passage.

|| Undiluted in 196 Fb-L.

tained by serial subculture. Meanwhile, in parallel cultures, polygonal cells did not appear, and cells serially subcultured from these have retained their fibroblast-like morphology for over a year. This phenomenon offered an opportunity to study viruses simultaneously in 2 different morphologic types of cells derived from the same source. Table I lists the origin of each Detroit cell strain, pertinent data on the human donor and the period during which each strain has been maintained.

Viruses. The ability of ECHO viruses,† types 1 through 16, to produce cytopathogenic changes or to multiply in each of the de-

scribed cell strains was investigated. Tube cultures of each of the cell strains were prepared by implantation with approximately 1×10^5 cells in 1 ml of the growth medium. The Ep-L strains formed cellular monolayers in 2 days, the Fb-L strain in 5-6 days. Each virus (in previously assayed fluids from infected monkey kidney cultures) was diluted as indicated in Table II, and 0.1 ml layered on the cells of each of 2 cultures. After an

† The prototype ECHO viruses were kindly furnished by J. L. Melnick, W. McD. Hammon, and from the laboratory of A. B. Sabin by M. Ramos-Alvarez.

TABLE III. Behavior of 16 ECHO Viruses in Cultures of 5 Human Epithelial-Like Strains.

| ECHO virus type | TCD ₅₀ * in M.K. cultures | Dilution of inoculum | Passage† in human cell strains | | | | | | | | | | | |
|-----------------|---|----------------------|--------------------------------|---|---|------------|---|---|------------|---|-----|--------------|---|-----|
| | | | Detroit-30A | | | Detroit-32 | | | Detroit-98 | | | Detroit-116P | | |
| | | | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 7.2 | 1:100 | — | — | — | + | — | — | — | — | — | + | + | 4.6 |
| 2 | 6.8 | " | + | — | — | + | — | — | — | — | — | + | — | — |
| 3 | 6.2 | " | + | — | — | + | — | — | + | — | — | + | + | 5.2 |
| 4 | 7.5 | " | + | — | — | + | — | — | + | — | — | + | — | — |
| 5 | 7.5 | " | + | — | — | + | — | — | + | — | — | + | — | — |
| 6 | 6.8 | " | + | + | — | + | + | — | + | — | — | + | + | 6.3 |
| 7 | 6.6 | " | + | + | — | + | — | — | + | — | — | + | + | 3.6 |
| 8 | 5.6 | " | — | — | — | — | — | — | — | — | — | + | + | 4.4 |
| 9 | 4.5 | " | — | — | — | + | — | — | — | — | — | + | — | — |
| 10 | 4.5 | " | + | — | — | + | — | — | + | — | — | + | + | 1.6 |
| 11 | 5.7 | " | + | — | — | + | — | — | + | — | — | + | — | — |
| 12 | 6.5 | " | + | — | — | + | — | — | + | + | 2.6 | — | + | 1.6 |
| 13 | 6.4 | " | — | — | — | — | — | — | + | — | — | + | + | — |
| 14 | 7.3 | " | + | + | — | + | — | — | + | — | — | — | — | — |
| 15 | 1.5 | Undiluted | — | — | — | — | — | — | — | — | — | — | — | — |
| 16 | 4.5 | 1:100 | — | — | — | — | — | — | — | — | — | — | — | — |

* Expressed as log dilution/0.1 ml.

† As determined by CPE or back titration in monkey kidney cultures.

adsorption period of 15-20 minutes, 1.0 ml of BME containing 5% calf serum was added to each inoculated culture as well as to uninoculated control cultures. This medium contained no inhibitors for the 16 ECHO viruses. All cultures were incubated at 35°C and were observed daily. As soon as definite cytopathogenic effects occurred, the culture fluids were removed and frozen. If cytopathogenic effects were not apparent, the culture fluids were frozen at the time that the uninoculated control cultures showed evidence of degeneration. Except where noted in Tables II and III, such fluids were centrifuged, diluted 1:100, and similarly carried through 2 additional passages in each cell strain. Where indicated, the culture fluids collected at each passage were tested undiluted, or back-titrated, in monkey kidney cultures for presence or titer of virus.

Results. Table II shows that each of the ECHO virus types, with the single exception of ECHO type 10, was cytopathogenic for Detroit-196 Fb-L cells. In first passage in these cells, 15 ECHO virus types produced distinct cytopathogenic effects within 1-3 days and usually all of the cells in the cultures were affected within the next 1-2 days. These intervals were generally shortened in 2 successive serial passages, but throughout the 3 passages on the Detroit-196 Fb-L strain, it

was noted that ECHO types 7 and 11 consistently produced cytopathogenic effects more rapidly than the others, whereas ECHO type 15 was consistently slower. The culture fluids recovered from the 3rd serial passage were back-titrated in monkey kidney cultures. The titers, listed in Table II, show that there is no doubt that multiplication of virus had taken place. No attempt was made to ascertain the significance of differences in these titers before and after 3 passages in the Detroit-196 Fb-L strain. The failure of ECHO virus type 10 to proliferate in these cells did not appear to be due to the pH of the culture fluids or to the lability of the virus as has been shown in the case of monkey kidney cultures(6,7). However no multiplication of ECHO virus type 10 could be demonstrated in Detroit-196 Fb-L cells when the medium was maintained either at pH 7.2 or at pH 8.0.

In contrast to their fibroblast-like counterpart, the Detroit-196 Ep-L strain appeared susceptible to only 2 of the ECHO viruses, type 6 and type 10. Cytopathogenicity was difficult to determine in these particular epithelial-like cells, partly because this strain does not form confluent monolayers on glass and partly because uninoculated control cultures often exhibited areas of cellular degeneration during the test period. Consequently, culture fluids of the first virus passage were

transferred undiluted to monkey kidney cultures, and cytopathogenicity in the latter served as an indicator system. As shown in Table II, 12 of the ECHO viruses were present in Detroit-196 Ep-L cells at the end of one passage. Culture fluids were then "blindly" passed through a second passage. Of the third passage culture fluids, only those of the ECHO type 6 and type 10 series produced cytopathogenic effect in monkey kidney cells, and these at dilution end-points of $10^{-3.2}$ and $10^{-2.5}$, respectively. The total dilution through 3 passages makes it likely that these titers represent some multiplication and are not due to residual inocula.

Table III illustrates the behavior of the 16 ECHO viruses in epithelial-like strains, Detroit-30A, -32, -98, -116P, and HeLa. In the Detroit strains, most of the viruses had disappeared by the end of the second passage. ECHO virus type 12 was present in low titer in the third passage culture fluids of Detroit-98, and ECHO type 10 in appreciable titer in third passage fluids of Detroit-116P. Of the Ep-L strains tested, HeLa appeared to be susceptible to the greatest number of ECHO virus serotypes, namely types 1, 3, 6, 7, 8 and possibly types 10 and 12, although multiplication of the latter 2 types might be questioned because of the very low titers.

Discussion. Surveys of the virus susceptibilities of human cell strains have been carried out in this laboratory with a dual purpose in mind: to further characterize cells of similar morphology but perhaps differing in other biologic properties, and also to determine whether or not various cell strains might be useful for the cultivation of viruses hitherto handled inconveniently in the laboratory. Studies of the comparative behavior of viruses in human cell strains have usually involved epithelial-like (Ep-L) cells(8-13). One is impressed that such cell strains, irrespective of their tissue sources, are more or less uniformly susceptible to a wide variety of animal viruses and uniformly refractory to others.

Recently it has been shown that many human cell strains now in existence might be classified into 2 groups on morphologic grounds: a) Ep-L cells, of which the HeLa

strain may be taken as the prototype; and b) the more recently described "smaller round or irregularly shaped" cells that "do not form islands or sheets unless cultures become crowded"(14). These differences in cell morphology are matched by marked differences in their viral spectra(13). In addition, certain human fibroblast-like (Fb-L) strains are susceptible to viruses that have not been previously propagated in Ep-L strains(15). Thus it appears that differences in morphology are of significance with respect to virus susceptibility. This concept is emphasized by the present study where it is shown that a human Fb-L strain differs markedly from human Ep-L strains in its susceptibility to ECHO viruses. Detroit-196 Fb-L was susceptible to all but one of the ECHO virus serotypes whereas among 6 human Ep-L strains, 2 were completely refractory, and 4 had a narrow and variable susceptibility range.

The wide susceptibility range of the Detroit-196 Fb-L strain is of practical importance since it is an example of a cultured human cell type that may be useful for studies of ECHO viruses.

The marked differences in ECHO virus susceptibility between the Detroit-196 Fb-L strain and its Ep-L derivative would be of particular interest if it could be determined whether or not the Fb-L cell had undergone a transformation of the kind described in detail by Berman, *et al.*(16), or if the Ep-L cell was selected from a mixed cell population by a mechanism similar to that proposed by Puck, *et al.*(17).

Within the group of Ep-L cell strains themselves, there were differences in susceptibility. These are the first marked differences we have seen between these particular Detroit Ep-L strains other than minor morphologic variations.

Summary. The susceptibility ranges of a fibroblast-like and 6 epithelial-like human cell strains to 16 ECHO virus types have been described. All of these ECHO viruses except type 10 multiplied in the fibroblast-like cell strain (Detroit-196 Fb-L). In contrast, an epithelial-like strain (Detroit-196 Ep-L) evolving from the Fb-L strain, as well as 5 additional Ep-L cell strains, either were re-

fractory or had narrow susceptibility ranges. The significance of differences in morphology with respect to virus susceptibility was discussed.

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Fiber Formation in Suspension Cultures of L Strain Fibroblasts.* (23743)

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While numerous workers have reported fiber formation in primary explant cultures of fibroblasts, as indicated in reviews by Bloom and Fell(1,2) there have been no such reports concerning monolayer or suspension cultures of stable cell strains. In this paper, conditions are described under which L strain mouse fibroblasts in agitated cultures give rise to membranous structures containing numerous discrete fibers. A preliminary study of the origin and nature of these fibers is reported herein.

Materials and methods. Strain L 929 fibroblasts(3) were maintained in stationary bottle cultures in synthetic mixture 199† supplemented with 1.0% Bactopeptone‡ (199P). To prepare suspension cultures cells were scraped from the glass, dispersed by vigorous

pipetting and diluted to a concentration of $1.5-2.5 \times 10^5$ cells/ml in medium 199P. One hundred milliliters of the suspension were put into a 250 ml Erlenmeyer flask. Control cultures were prepared by suspending an equal number of cells in medium 199P supplemented with 5% horse serum (199P-HS). Both the experimental flasks and the controls were stoppered and placed on a rotary action shaker§ (100 rpm) at 35°C(4). After several hours incubation delicate membranes appeared on the surface of the serum-free medium. A number of these membranes were pipetted onto cover glasses. Such preparations were fixed either in Zenker-acetic or in 10% formalin, and were stained with Regaud's modification of the Masson trichrome technic or by Foote's modification of the Bielschowsky silver method for argyrophilia (5). Membranes from several flasks were also collected by centrifugation and fixed in 10% formalin. These were minced in a Waring blender and the resulting material

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† Microbiological Associates, Bethesda, Md.

‡ Difco Laboratories, Detroit, Mich. Lot No. 431352.

§ Eberbach and Co., Ann Arbor, Mich.

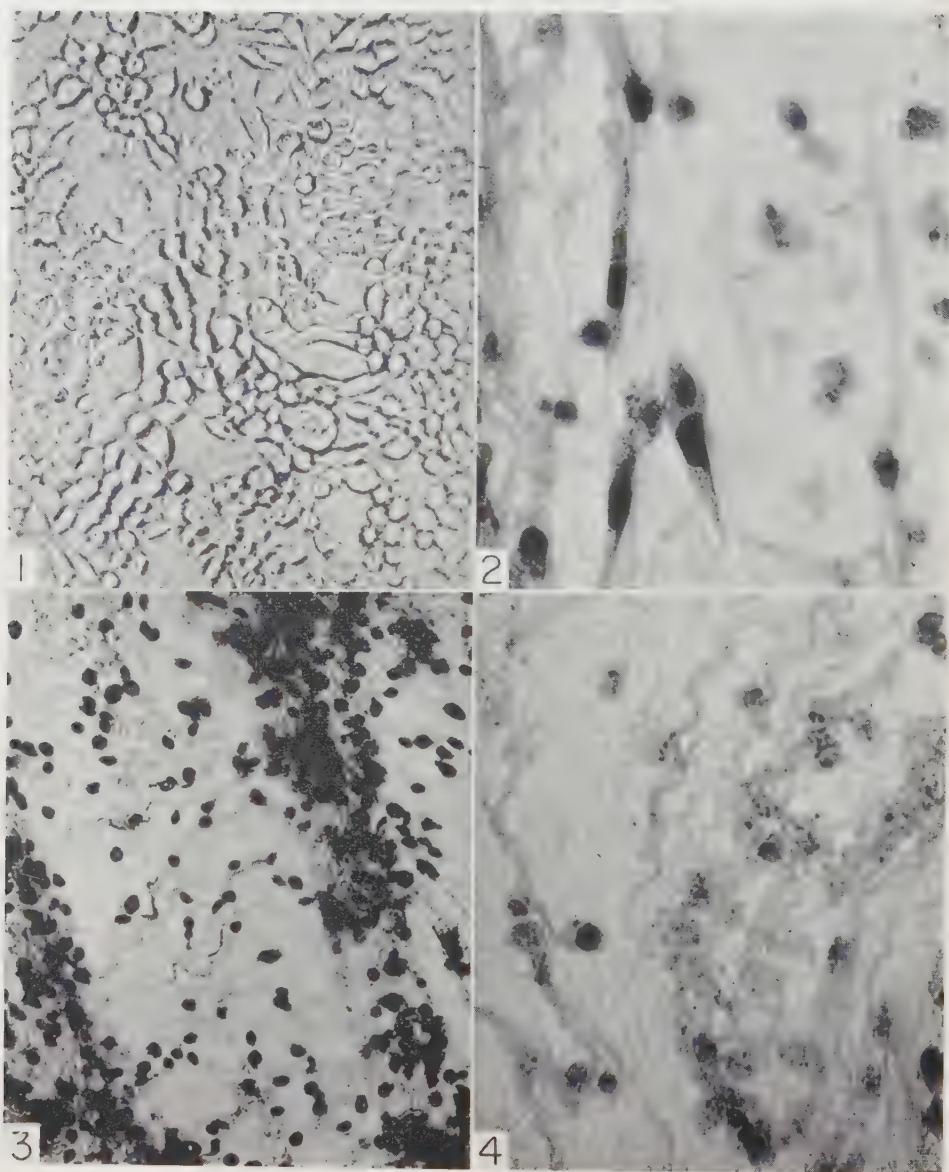


FIG. 1. L strain fibroblasts grown as a monolayer in medium 199 supplemented with 0.5% Bactopectone. Unstained. $\times 300$.

FIG. 2. L strain fibroblasts grown in suspension culture in medium 199 with 1% Bactopectone. Haematoxylin and eosin. $\times 700$.

FIG. 3. L strain fibroblasts grown in suspension culture in medium 199 with 1% Bactopectone. Silver stain. $\times 300$.

FIG. 4. L strain fibroblasts grown in suspension culture in medium 199 with 1% Bactopectone. Masson stain. $\times 700$.

was used to prepare grids for electron microscopy. Such specimens were treated with 5% phosphotungstic acid at pH 5.6 for $\frac{1}{2}$ minute, washed, dried, and treated with 1% phosphotungstic acid at pH 3.5 for 1 minute(6). Control preparations were made by fixing rat-

tail tendon in 10% formalin and following through with the preparation of grids for electron microscopy as already outlined.

Results. L strain fibroblasts grown on a glass substrate in medium 199P with an initial inoculum of 2×10^5 cells/ml yielded a

monolayer of spindle shaped cells in 10-14 days (Fig. 1). The generation time during logarithmic growth was approximately 50 hours and the maximum population density was $1.5-2.0 \times 10^6$ cells/ml. The addition of serum to the medium (199P-HS) shortened the generation time (35-40 hours) but did not significantly alter either the growth pattern or the maximum cell density. Stationary bottle controls of L strain in medium 199P exhibited cells trapped at the liquid-air interface which grew there much as they did on glass. The cells extended in a spindle-shaped fashion and were attached to one another laterally by long processes. Often these cell masses were quite extensive and floated as fairly rigid units.

In an agitated fluid suspension, the addition of 5% whole horse serum to the medium (199P-HS) permitted the rapid proliferation of L strain fibroblasts. Under these conditions the population developed as an even suspension of cells which were essentially spherical. The generation time in such cultures was 26-30 hours and the maximum population density approached or even exceeded 2.0×10^6 cells/ml. When, however, the cells were suspended in medium 199P and agitated on a rotary action shaker, delicate membranes were observed to form at the fluid-air interface within 24-48 hours and continued to float on the surface of the medium. Microscopic examination of such membranes revealed pleomorphic cells dispersed among large numbers of fibers arranged singly and in bundles (Fig. 2, 4).

Continued agitation of the cultures caused the membranes to fold and become submerged as they increased in diameter. However, when flasks were removed from the shaker after membrane formation had begun and incubation was continued under static conditions, the membranes continued to increase in diameter, ranging up to 5 cm. Staining of these older membranes with silver-staining procedures demonstrated argyrophilic fibers (Fig. 3) and when the Masson trichrome technic was used the fibers stained a deep blue. The intensity of argyrophilia could be increased by immersing the membranes in

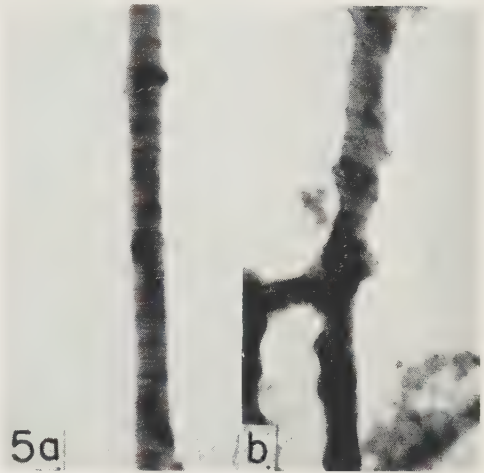


FIG. 5a. Electron micrograph of collagen fibril from rat-tail tendon. Phosphotungstic acid stain. $\times 55,000$.

FIG. 5b. Electron micrograph of fibril from L strain culture grown in medium 199 with 1% Bactopeptone. Phosphotungstic acid stain. $\times 55,000$.

dilute joint fluid prior to staining. This procedure also intensified the staining of fibers in young membranes by the Masson technic. The cells in these membranes demonstrated a gradation of morphology and staining properties. Although the rounded cells in suspension ranged from 12μ to 18μ in diameter these spindle-shaped cells found in the membranes ranged from 18μ to 145μ in length. With the Masson technic, they varied in tinctorial properties from smaller cells exhibiting a lightly stained nucleus and fine eosinophilic granules in the cytoplasm to the larger cells displaying a deeply stained nucleus and coarse cyanophilic cytoplasmic granules.

In an effort to determine the nature of the fibers, preparations were examined with the aid of an electron microscope. When standard procedures were followed, fibrils were obtained from the membranes which had major periodicity of 640 A (Fig. 5). A control preparation of a fibril from rat-tail tendon is shown for comparison. The fibrils from the membranes were digested by collagenase[†] from *Clostridium perfringens* and were par-

[‡] Courtesy of Dr. William Castor, Rackham Arthritis Unit, Univ. of Michigan.

[†] Courtesy Dr. John McLennan, Dept. of Microbiology, Columbia University.



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FIG. 6. Electron micrograph of fibril from L strain culture grown in medium 199 with 1% Bactopeptone, treated with dilute acetic acid. Phosphotungstic acid stain. $\times 55,000$.

tially degraded by trypsin, but were resistant to α amylase and hyaluronidase. The fibrils swelled when treated with acetic acid (Fig. 6).

Discussion. At least 3 explanations of the observed facts seem plausible: (a) In a medium which is suboptimal for growth, many cells may degenerate, freeing fibrous material which accumulates at the liquid-air interface and entraps other free cells; (b) under all conditions of growth, the cells may produce soluble proteins which denature at the liquid-air interface contingent upon the methods described here; (c) under all culture conditions, the cells may synthesize a fibrous protein which is intimately associated with the cell surface though not necessarily a part of the cell membrane. When cells aggregate at the liquid-air interface, are bound together laterally and undergo tension as the result of agitation, this fibrous protein may be protracted and entwined in strands. With regard to the latter possibility it is of interest to note that when L-strain cell suspensions are treated with 0.1 M citric acid, a fibrous material is rapidly shed from the cell surface. This shedding of material does not immediately affect

the cell membrane which remains intact and only later ruptures suggesting a labile protein surface (unpublished). Further evidence of the existence of a superficial proteinaceous material related to the binding of cells to one another and to a solid substrate is indicated by the fact that trypsin and other proteolytic enzymes are effective in the dispersal of tissue cells *in vitro*.

It is of some interest that the fibrogenic capacities exhibited by the L strain cells are not unique to this strain but have been duplicated in our laboratory using the LLC-M1 strain of mouse fibroblasts isolated by Hull(7).

Summary. The *in vitro* formation of fibrous membranes by the L and LLC-M1 strains of mouse fibroblasts in a protein-free medium has been demonstrated. When cells of either of these strains are suspended in medium 199 plus 0.5-1.0% peptone and incubated at 35°C on a rotary action shaker at 100 rpm, delicate membranes form at the liquid-air interface. Enzymatic digestion, staining reactions and electron micrographs suggest that the fibers are fibrous protein in nature and belong to the collagen class of protein. The possible relationship of this fibrous protein to the cell surface is discussed.

We are indebted to Mrs. Frances Loranger for preparation of the electron micrographs.

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Intracellular Glycogen and Electrolyte Concentrations in Human Skeletal Muscle.* (23744)

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Values for less than 100 analyses of human skeletal muscle for electrolytes are available in the literature(1-7), and, of these, the largest series was conducted on post-mortem tissues(1). Furthermore, calculation of the intracellular concentrations of ions was carried out by the authors in only 3 series(4,5,7). The only data available on the glycogen content of human skeletal muscle is that of Moscati(8). The present communication presents the intracellular concentrations of sodium, potassium, phosphate, and glycogen obtained in 6 normal human subjects and discusses their possible interdependence.

Methods and calculations. Patients entering the hospital for elective surgical procedures were selected for biopsy studies. Specimens were obtained after the induction of anaesthesia and following the administration of 0 to 400 cc of 5% dextrose intravenously. Blood samples for the determination of water, chloride, sodium, and potassium were drawn one to four hours before the biopsy specimens were obtained. Of 11 patients in whom biopsies were carried out, 5 patients had abnormal serum electrolyte values and are not included in the study. Biopsy

specimens weighing 1-2 g were obtained from the rectus abdominis muscle after induction of anaesthesia and before any other surgical procedures had been carried out. Two hundred to 400 mg of tissue were immediately placed in a tared tube containing 2 cc of 30% KOH for glycogen analysis. The remainder of the specimen was placed in a tared weighing bottle, brought to constant weight at 100°C., fat-extracted, and the dry, fat-free tissue analyzed for sodium, potassium, chloride, and phosphate. The methods used in the analysis of the tissues for glycogen, and the plasma and tissues for fat, water, and electrolytes have been previously described by the author(9,10). Calculations of the intracellular concentrations of electrolytes were made assuming the extracellularity of chloride, using the formulae of Hastings and Eichelberger(11). It is unfortunate that blood samples were not obtained simultaneously with biopsy specimens, as this may have introduced errors into the calculations of intracellular values. However, this was not possible.

Results. Table I presents the ages, clinical diagnoses, type of anaesthesia, and amount of

TABLE I. Clinical Data on Patients.

| Patient, age and sex | Dx | Operation | Anaesthesia | 5% dextrose, cc |
|----------------------|--|---|------------------------|-----------------|
| (1) M.O. 64 ♂ | Duodenal ulcer | 2nd stage subtotal gastric resection | Cyclopropane and ether | 0 |
| (2) M.R. 49 ♀ | Carcinoma of sigmoid | Left colectomy | <i>Idem</i> | 400 |
| (3) S.R. 50 ♀ | Pericolic abscess following exploratory laparotomy | Excision of abscess; closure of colostomy | " | 210 |
| (4) P.M. 77 ♂ | Carcinoma of sigmoid | Resection of sigmoid | GOE | 100 |
| (5) H.A. 55 ♀ | Abdominal carcinoma | Exploratory laparotomy | Cyclopropane and ether | 150 |
| (6) G.C. 60 ♂ | Adenocarcinoma of sigmoid | Resection of sigmoid | <i>Idem</i> | 140 |

*Supported by Public Health Research Grant H-748.

TABLE II. Basic Data.

| Patient | Plasma | | | | Muscle* | | | | | |
|---------|-----------------------|-------------------------|-----------------|----------------|-------------------------------|--------------------------------------|-----------------|------|---------------------------------------|---------------------------|
| | H ₂ O, g/l | Cl ⁻ , meq/l | Na ⁺ | K ⁺ | H ₂ O, g/kg tissue | Cl ⁻ meq/100 g dry solids | Na ⁺ | K | PO ₄ , mM/100 g dry solids | Glycogen, g/kg wet tissue |
| 1 | 936 | 102 | 149 | 3.9 | 778 | 11.0 | 16.5 | 42.4 | 23.2 | 5.28 |
| 2 | 940 | 98 | 150 | 4.1 | 786 | 10.0 | 16.8 | 42.2 | 25.1 | 15.68 |
| 3 | 942 | 104 | 154 | 4.2 | 789 | 15.0 | 24.5 | 39.7 | 23.5 | 9.50 |
| 4 | 938 | 100 | 143 | 4.0 | 778 | 7.3 | 14.6 | 46.1 | 27.8 | 13.70 |
| 5 | 938 | 104 | 149 | 3.6 | 788 | 14.3 | 22.4 | 38.4 | 23.0 | 8.62 |
| 6 | 936 | 100 | 146 | 3.9 | 787 | 9.6 | 16.2 | 43.8 | 25.9 | 15.74 |
| Mean | 938 | 101 | 149 | 4.0 | 784 | 11.2 | 18.5 | 42.1 | 24.8 | 11.42 |
| S.D. | 2.2 | 2.5 | 3.5 | .2 | 4.9 | 3.2 | 4.0 | 2.8 | 1.9 | 4.2 |

* All data are expressed in terms of fat-free tissue.

5% dextrose received by the patients. Table II presents the basic data for plasma and muscle in the 6 subjects, together with the mean and standard deviation for each set of values. Plasma water and electrolyte values were all within the range of normal. In the case of the basic values for muscle, there was a wide variation in the values for chloride and sodium, representing considerable differences in the size of the extracellular spaces in the samples. The values for water, potassium, and phosphate fell within a close range. Potassium and phosphate concentrations tended to vary inversely with the concentrations of chloride and sodium. Glycogen content varied from 5.28 to 15.74 g/kg wet tissue.

Table III presents the chloride spaces and the concentrations of sodium, potassium, phosphate, and glycogen in the intracellular water of the muscle cells of the 6 subjects. The chloride spaces ranged from 18.7% of total muscle water to 33.4%. This should not be interpreted as a normal variation for two reasons: (1) the connective tissue content of the samples varied considerably, and (2) all but

1 patient was receiving intravenous fluid at the time of biopsy. Average values in connective-tissue free, normal skeletal muscle are probably lower than the average 27% of total muscle water found in these samples. The values obtained for intracellular phosphate were quite constant, varying only from 90.2 to 95.6 mM/kg of cell water, with a mean value of 92.0. The concentration of intracellular potassium ranged from 152 to 165 meq/kg of cell water, with a mean of 156. Intracellular sodium showed values of from 7.4 to 16.7 meq/kg of intracellular water. Glycogen constituted from 2.4 to 6.8% of the total tissue solids, and was present at concentrations of from 9.3 to 26.6 g/kg of intracellular water.

Discussion. The values obtained by Moscati for the glycogen content of human skeletal muscle ranged from 0.4 to 0.6 g %. These values are considerably higher, and show a greater range. They are similar to values found in rats by the author(12) and others (13). It is of interest to note that patient M. O., who had fasted for 14 hours and had

TABLE III. Derived Data.*

| Patient | Chloride space, | | Na (meq/kg intrac. H ₂ O) | K (meq/kg intrac. H ₂ O) | PO ₄ , mM/kg intrac. H ₂ O | Glycogen | |
|---------|---------------------------------|------------------|--|---|--|-----------------------------|----------------------------------|
| | g H ₂ O kg muscle | H ₂ O | | | | % total tissue solids | g/kg intrac. H ₂ O |
| 1 | 272 | | 7.4 | 165 | 90.2 | 2.4 | 9.3 |
| 2 | 250 | | 10.0 | 152 | 90.6 | 7.2 | 26.6 |
| 3 | 349 | | 16.6 | 161 | 95.6 | 4.4 | 18.5 |
| 4 | 187 | | 16.7 | 154 | 92.7 | 5.9 | 20.7 |
| 5 | 334 | | 13.7 | 153 | 91.9 | 3.9 | 16.5 |
| 6 | 232 | | 11.7 | 150 | 90.8 | 6.8 | 26.0 |
| Mean | 271 | | 12.7 | 156 | 92.0 | 5.1 | |
| S.D. | 68 | | 3.7 | 5.4 | 2.0 | 1.85 | 6.4 |

* All data are expressed in terms of fat-free tissue.

TABLE IV. Intracellular Ion Concentrations in Human Skeletal Muscle.*

| Ref. | Author | Date | No analyses | Na meq/kg ICW | K meq/kg ICW | P, mM/kg ICW |
|------|----------|------|-------------|---------------------|--------------------|--------------------|
| 1 | Cullen† | 1933 | 19 | | 121.0 | 92.0 |
| 2 | Shohl† | 39 | | 12.4 | 145.0 | 110.0 |
| 3 | Mangun | 41 | 13 | | 145.0 | 111.7 |
| 4 | Mudge | 49 | 3 | 8.6 | 157.0 | |
| 5 | Eliel | 51 | 6 | 11.4 | 134.3 | 85.7 |
| 6 | Baldwin | 52 | 15 | | 164.0 | 101.6 |
| 7 | Mokotoff | 52 | 4 | 11.4 | 157.0 | 101.0 |
| 8 | Nichols | 57 | 6 | 12.7 | 156.0 | 92.0 |

* Where values for total water, ECF, and/or plasma values were not given, the mean values obtained in this series were used to calculate the (ion)_i.

† Post-mortem analyses.

‡ Compiled from literature.

received no glucose had maintained a muscle glycogen content of 0.528 g %. This maintenance of relatively high muscle glycogen levels despite fasting has also been demonstrated in rats by the author. In ten rats fasted 24 hours, muscle glycogen fell only from 0.86 to 0.40 g %. This was in contrast to liver, where glycogen concentrations decreased from 7.43 to 0.13 g % in the same period. There did not appear to be a quantitative correlation between the amount of glucose that had been administered to the patients and glycogen levels in the muscle. Patient M.R. who had received 400 cc of 5% glucose and patient H.A. who had received only 150 cc had almost identical muscle glycogen concentrations. This may have been related to the blood sugar levels which were not determined, or may be due to the individual reactions to the varying stresses to which these patients were subjected.

Table IV presents the data obtained by other workers for intracellular ion concentrations in human skeletal muscle. There is surprisingly good accord between the average values for intracellular sodium, although the individual values in each series show a wide range. Five of the 8 studies show an average intracellular potassium concentration of 145 to 157 meq/kg intracellular water, with 2 series (1 and 5) having lower values, and one (6) higher. The extreme values for total intracellular phosphate are 85.7 to 111.7 mM/kg intracellular water, with a mean value of 99 for all of the series.

A number of studies have been made which indicate that the deposition of glycogen is accompanied by a fall in the serum concentrations of potassium and phosphate(14,15,16). Conversely, it has been shown that potassium and phosphate administration can lower the blood sugar(17,18) and prevent the occurrence of the hyperglycemia occurring from glucose administration following fasting(19). There is good evidence in animals that the deposition of glycogen in liver is accompanied by the deposition of potassium(20), which would account for the observed fall in serum levels of this ion. Whether or not potassium also enters muscle during glycogen deposition is not known. Phosphate, on the other hand, does not enter liver cells when glycogen is deposited(9), the level of total liver phosphate declining as liver glycogen rises. Clinical evidence confirms this and suggests(21) that the fall observed in serum phosphorus after glucose administration is due to the entry of phosphate into skeletal muscle(21,22). It seemed of interest therefore to study the correlation between the levels of intracellular muscle glycogen and potassium and phosphate in these 6 patients. Although the glycogen levels ranged from 5.28 to 15.74 g/kg of wet tissue, or 9.3 to 26.0 g/kg of intracellular water, representing a broad physiological span(23), glycogen constituted only 2.4 to 6.8% of the total tissue solids. Assuming muscle glycogen to be deposited with the same amount of water as is found in association with nitrogenous solids(20), the addition of 10.46 g of glycogen (*i.e.* a rise from 5.28 to 15.76 g/kg of wet tissue) to a kilogram of tissue would only command the addition of 7.96 meq of potassium and 4.7 mM of phosphate to maintain the pre-existing level of these ions in the intracellular water. Conversely, non-entry of these ions would only cause a fall in concentration of 7.96 meq of potassium or 4.7 mM of phosphate. It is apparent that these changes are very small, and unlikely to be statistically verifiable in this small series. The correlation coefficient for the addition of potassium to the intracellular water of skeletal muscle for these 6 tissues is -0.776 , which is suggestive that potassium concentrations fall as glycogen is added to skele-

tal muscle. The standard error of the estimate for this small series shows that there is an 84% chance that this correlation did not occur by accident, but there is less than the 95% chance which is considered the lower limit of significance. It must be borne in mind that these patients were receiving glucose without exogenous potassium, and that the body has no known reservoir for this ion. Had exogenous potassium been available, as is the case in the normal diet, potassium levels in muscle cell water might have remained constant. In contrast to the relation between potassium and glycogen the correlation coefficient for intracellular phosphorus and glycogen is -0.027 (no correlation), indicating that the concentration of phosphorus in the intracellular water of skeletal muscle does not fall during the deposition of glycogen. Again assuming the addition of intracellular water to muscle mass as glycogen is deposited, this suggests that phosphate ion is added to skeletal muscle cells in order to maintain a constant level of this ion, even in the absence of an exogenous supply of this ion.

Summary. The composition of human skeletal muscle has been examined in 6 normal individuals. Intracellular concentrations of sodium, potassium and phosphate have been calculated and agreed closely with values reported by other workers. Glycogen levels ranged from .528 g % in a fasted patient to 1.57 g % in a patient who had received glucose. Glycogen levels were not directly correlated with the amount of glucose given. There is some evidence (correlation coefficient of -0.776) that in the absence of exogenous supplies, potassium is not deposited in muscle cells when glycogen is deposited, whereas phosphate may be deposited (correlation coefficient of -0.027).

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Persistence of Taste Organs in Tongue Grafted to Liver. (23745)

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It has previously been recorded that taste organs of the adult newt (*Triturus v. viridescens*) can persist for at least a year without benefit of any trophic action from gustatory nerve fibers(1). This information was derived from histological examination of taste organs in tongue-tips grafted to orbit. The sense organs are effectively divorced from gustatory nerve connections in such grafts, but there is reason to believe that other nerve fibers of central origin grow into the tissues. For example, the striated muscle of the tongue does not atrophy. It was considered possible, therefore, that a trophic influence from invading, non-specific, nerve fibers could play a role in maintenance of structure of the taste organs deprived of specific nerve fibers. In order to denervate the grafts more completely, tongue-tips were transplanted to the liver. It seems most unlikely that there can be any innervation to the graft tongue tissues other than by autonomic fibers such as those associated with blood vessels. This is borne out to some degree by the fact that striated muscle atrophies in these grafts.

Methods. Autoplastic transplantations were performed on 111 newts as follows. With the animals under chlorotone anesthesia, a small incision was made in the ventro-lateral body wall just posterior to the axilla on the right side; the surface of the liver, well anterior to the incision, was scratched lightly to provide a suitable graft site; the tip of the tongue was excised, inserted through the incision, and released in the vicinity of the scratched liver; and, finally, the edges of the incision were pinched together. All animals were kept nearly motionless in cool moist-chambers until the incision had sufficiently healed to warrant their being placed in aquaria. Of the 57 transplants eventually recovered, 43 were attached only to the liver, 8 were connected

to both liver and body wall, and 6 remained free in the body cavity. Grafts were recovered at various time intervals after transplantation, but the primary object of the present account is to report only on the 11 cases examined histologically after 4 months.† All these grafts were well vascularized.

Results. The grafts range in size from one to 2.5 mm in diameter. The number of taste organs on the surface of the grafts ranges from none to 63, but there is no direct relation between the number of taste organs and the size of the graft. There are taste organs of normal structure in 8 of the 11 cases.

The meaningful correlation, apparently, is between the number of sense organs and the area of *normal* tongue epithelium, but the character of the surface epithelium is highly variable. Three of the grafts are covered by a flat, membranous epithelium. These have no taste organs though 2 of them are as large as another graft possessing 63 organs. The latter, however, is entirely covered by a normally thick epithelium. Another graft of comparable size has 19 organs, and its epithelium is partly of normal thickness and partly membranous. One smaller graft is primarily covered by a membranous epithelium, but it has a small patch of thick epithelium which supports three sense organs. The remaining five grafts contain combinations of the normal, the membranous, and a highly glandular and ciliated type of epithelium. The numbers of sense organs in these (15, 2, 7, 6, 9+) also vary according to the area of normal epithelium.

It is impossible to know in these experiments at what time after operation the grafts became attached to the liver. It seems a reasonable assumption that there was considerable variation in this respect. It, therefore, may follow that the epithelium in those

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cases taking a relatively longer time to establish vascular connections began to degenerate, and that the variation in the number of sense organs in these grafts is a reflection of that circumstance. The fact that all "grafts" which remained free in the body cavity were covered only by membranous epithelium containing no sense organs lends support to this interpretation.

At any rate, the experiment clearly indicates that taste organs can be maintained in the newt for at least 4 months without any trophic influence from nerve fibers of central origin in their immediate vicinity.

Summary. Tongue-tips were transplanted autoplastically to the liver in *Triturus v. viridescens* to effect denervation of the taste organs. All well-vascularized grafts that re-

tained a normally thick epithelium contained histologically normal taste organs when examined 4 months after transplantation. This fact indicates that these taste organs were independent of any trophic action from nerve fibers of central origin. The number of persistent taste organs is correlated with the amount of normal epithelium. It is suggested that differences in the time of reestablishment of circulation in the grafts may account for the variation found in the type of epithelium (thin, membranous or normal) and, therefore, may also account for the variation in number of taste organs persisting.

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Respiration of Rat Liver Homogenates Following Prolonged Cold Exposure. (23746)

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Although it has been fairly well established (1) that cold exposure leads to an increased tissue oxygen consumption, relatively little is known about the factors responsible for this increase. Work with tissue slices, as shown previously (2), is uninformative in this regard, since the rates observed are for the most part indicative only of endogenous respiration. The work that follows was undertaken in order to elucidate some of the factors responsible for the changes observed with slices. To this end, a variety of substrate oxidations were tested in control and in cold-exposed animals with a period of exposure which has been shown previously (2) to yield a maximal respiratory response in slices.

Methods. Male rats of the Sprague-Dawley strain were used in all experiments. They weighed between 225 g and 275 g at the time of selection. By the time of sacrifice for tissue studies, they were in the range of 275 to 350 g. The selection was made to obtain

groups of rats of similar initial weight, being subjected to cold exposure and acting as controls. The exposed animals were placed in individual cages in a cold room maintained at $5 \pm 1^\circ\text{C}$. for intervals of 29 to 33 days, whereas the controls were maintained in the animal colony at about 26°C . Both the control and experimental groups received a diet of "Friskies" dog food and water, given *ad libitum*, up to the time of sacrifice.

Tissue preparation consisted of a liver homogenate made up as follows: The animal was first killed by blow on head and the liver quickly excised and chilled in ice-cold 0.25 M sucrose. Utilizing a Potter-Elvehjem type homogenizer (3), a 10% homogenate was prepared from a portion of the large median lobe. This was carried out in ice-cold 0.25M sucrose containing 0.01M ethylenediamine-tetraacetic acid (pH 7.4). After filtering through 4 layers of cheesecloth to remove any large-size tissue fragments that may have been present, the homogenate was diluted to 3 1/3% with

TABLE I. Liver Homogenate Oxidations following Prolonged Cold Exposure.

| Substrate | Control | | Cold exposed | | % increase in cold | P |
|-------------------|----------------|-------------------|----------------|-------------------|--------------------|-----|
| | No. of animals | qO ₂ * | No. of animals | qO ₂ * | | |
| None | 15 | 53.6 ± 1.40‡ | 12 | 58.1 ± 1.53‡ | 8.4 | .05 |
| Lactate | 8 | 20.0 ± .70 | 7 | 20.9 ± 1.68 | 4.5 | .5 |
| Citrate | 15 | 142.1 ± 3.73 | 12 | 169.7 ± 3.69 | 19.4 | .01 |
| Isocitrate | 7 | 100.9 ± 6.90 | 6 | 135.1 ± 8.09 | 33.9 | .02 |
| α-Ketoglutarate | 15 | 145.3 ± 4.06 | 9 | 187.0 ± 5.09 | 28.7 | .01 |
| Succinate† | 8 | 208.2 ± 9.33 | 6 | 285.1 ± 8.70 | 37.2 | " |
| Fumarate | 12 | 94.6 ± 3.85 | 13 | 111.8 ± 2.42 | 18.2 | " |
| Malate | 4 | 96.6 ± 1.14 | 6 | 115.8 ± 1.53 | 19.9 | " |
| Glutamate | 15 | 153.1 ± 3.52 | 11 | 184.9 ± 5.90 | 20.8 | " |
| β-Hydroxybutyrate | 15 | 130.0 ± 3.80 | 9 | 173.3 ± 4.31 | 33.0 | " |

* The qO₂ values listed for each of the substrates have been corrected for endogenous respiration; magnitude of the latter appears under substrate heading of "None."

† qO₂ based on the first 10 min. of incubation.

‡ Mean ± S.E.

cold 0.25M sucrose containing 0.01M Tris (hydroxymethyl) amino methane buffer adjusted to pH 7.4 with KOH. From this final dilution, one milliliter aliquots were then placed in each reaction vessel. The tissue preparations were incubated at 38°C utilizing standard Warburg technique(3), allowing 10 minutes for thermoequilibration. The incubation medium had the following concentrations: 0.002M K-adenosinetriphosphate, (pH 7.4), 0.0066M MgCl₂, 1.5 × 10⁻⁵M cytochrome c, 0.0001M diphosphopyridine nucleotide, 0.01M substrate and 0.01M K-phosphate buffer (pH 7.4). The volume of the reaction mixture including the homogenate was adjusted to 3.0 ml in each incubation flask with 0.25M sucrose buffered at pH 7.4 with 0.01M Tris. All substrates were used as the potassium salts. In addition to the reaction medium, each vessel had 0.2 ml of 5N NaOH in the center well for CO₂ absorption and a gas phase of air. Rate of *tissue oxygen* consumption in the presence of each of the substrates was calculated on a per milligram nitrogen basis and reported as either qO₂ (μl O₂ consumed per mg N per hour) or as μl O₂ consumed per mg N for each 10-minute interval during the course of incubation. In the case of qO₂ measurements the values were corrected for endogenous respiration. Nitrogen was determined by nesslerization. Statistical comparison of the various groups was conducted by the group comparison method as outlined by Snedecor(4). In addition, standard error values were calcu-

lated for the mean qO₂ of each group.

Results. A summary of the data in the form of qO₂ values is given in Table I. Here it can be seen that in the absence of added substrate, the respiratory rate is significantly higher ($p < 0.05$) in cold-exposed animals than in controls. This result is in accord with those found previously(2) where the endogenous respiration of liver slices was measured. Table I also shows that all substrate oxidations, with the exception of lactate, proceeded at significantly higher rates in the cold-exposed group than in the control. In the presence of lactate, no difference was detected between the experimental and control groups. In the data on substrate oxidation, it can also be seen that 2 levels of increase are brought about by cold acclimatization. The first includes the oxidation of citrate, fumarate, malate and glutamate where the range of increase is from 18 to 21%; whereas the second includes isocitrate, α-ketoglutarate, β-hydroxybutyrate and possibly succinate where the range of increase is from 28 to 37%.

In addition to the increase in the qO₂ values exhibited by the cold-exposed groups, differences were also found in the pattern of oxygen uptake during the course of the incubation procedure. Examples of this are exhibited in Fig. 1 where the rate of oxygen uptake for successive 10-minute intervals of incubation is plotted against the elapsed time of incubation. The individual values in these curves represent the same number of animals

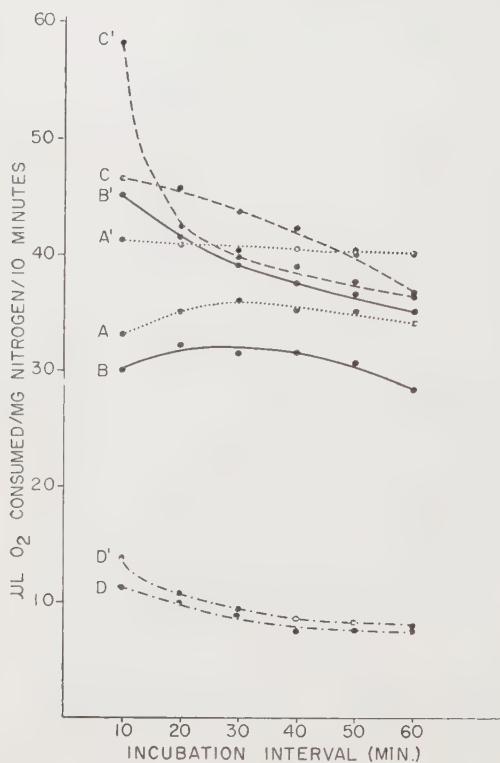


FIG. 1. Effect of duration of *in vitro* incubation on oxidation rates of liver homogenates from control and cold-exposed rats. Curves A, B, C and D represent the rates obtained in the presence of glutamate, β -hydroxybutyrate, succinate and no substrate, respectively, for control animals; A', B', C' and D' are the corresponding curves for cold-exposed animals.

as shown in Table I. The values have not been corrected for endogenous respiration, however.

It can be seen in Fig. 1 that when glutamate is being oxidized, the rate of oxygen consumption is relatively constant from the beginning until the end of incubation in both the control (curve A) and cold-exposed (curve A') groups. In contrast to this, the rate with succinate as substrate decreases very sharply during the first 10 minutes of incubation in the cold-exposed group (curve C') and then more slowly as the incubation is extended to one hour. Succinate controls (curve C) on the other hand, exhibit a more or less linear fall in rate over the entire period of incubation. It should be noted that the sharp initial drop in rate in the cold-exposed group carries the oxygen consumption below

the corresponding control values. Consequently, if a comparison of the two groups were made beyond the first 10 minutes of incubation, cold exposure could be interpreted as leading to a lowered rate of succinate oxidation. Measurements of rate change during the course of incubation when β -hydroxybutyrate was present as substrate (curves B and B') gave results that were more or less intermediate to those found with glutamate and succinate. The other substrates listed in Table I also exhibited rate changes during incubation that were quite similar to that shown for β -hydroxybutyrate. In no instance did the rate in the experimental group fall below the control level as was the case in curve C'. In fact, if all the qO_2 values had been calculated on the basis of the first 10 minutes of incubation as was done with succinate, the increases following cold acclimatization would have been much greater than those indicated in Table I.

Discussion. The foregoing data bring out 3 effects of cold acclimatization on *in vitro* liver metabolism: (a) an increase in the rate of endogenous metabolism, (b) an increase in the oxidation rate of a variety of metabolic intermediates, and (c) differences in the pattern of oxygen consumption during *in vitro* incubation.

Insofar as endogenous respiration is concerned, the cause of the increase in the cold-exposed group is unknown. In both the experimental and the control tissue the rates were low with respect to those found in the presence of added substrate. The increased rate could be due to a higher concentration of endogenous substrate in the cold-exposed group than in the control or to an increased enzyme activity or possibly to a combination of an increased enzyme activity and increased level of endogenous substrate. At least part of this question is resolved in the experiments where substrates were used.

As stated earlier, in all cases where substrate was added to the reaction medium, with the exception of lactate, there was a significantly higher qO_2 in the cold-exposed group than in the control. This indicates that cold exposure results in a more or less general increase in activity of the enzymes responsible

for aerobic oxidations. The data also indicate that the magnitude of the increase following acclimatization was greater with some substrates than in others. Since the substrates in the higher category are associated with dehydrogenase enzymes as the initial step in their breakdown, dehydrogenases of the tricarboxylic acid cycle may be more affected by cold exposure than other enzymes of this cycle. It should be noted that one of these in particular, succinic dehydrogenase, has been shown by others(5,6) to have an increased activity following cold acclimatization. However, it should also be noted in the present data that malate oxidation, although associated with a dehydrogenase, is not affected to the same extent by cold exposure as the other substrates associated with dehydrogenases.

Why lactate oxidation alone among all the substrates tested should show no difference between the control and the experimental values is not known. It may be related to the fact that lactate is associated with the soluble glycolytic system of enzymes whereas the other substrates are associated with the particulate enzymes of the tricarboxylic cycle. Both groups were found to have rates that were low in comparison to the other substrates. Therefore, it seems probable that homogenization and subsequent dilution of the tissues in the reaction vessels result in a loss of activity by one or more of those enzymes or cofactors responsible for the oxidation of lactate to the tricarboxylic acid cycle.

In the measurements of rate changes during the course of *in vitro* incubation, it was observed that the experimental tissue, in general, had a more pronounced fall than the

control tissue. This was especially true during the early stages of incubation. Two possible explanations might be offered for this phenomenon. The first is that the enzyme systems responsible for the oxidations are more labile following cold acclimatization. The second is that the products of the oxidations in the cold-exposed groups are accumulating at greater rates than in the controls and this in turn is leading to an inhibition of the oxidations themselves.

Summary. Respiration of liver homogenates derived from adult male rats exposed to cold for about one month was studied. Cold exposure led to 1) an increased endogenous metabolism and 2) increased rates when citrate, isocitrate, α -ketoglutarate, succinate, fumarate, malate, glutamate and β -hydroxybutyrate were present as substrates. No change in rate was observed when lactate was present as substrate. Differences were also found in both the magnitude and pattern of *in vitro* oxidations following cold exposure.

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Host Resistance to Hemorrhagic Shock. XI. Role of Deficient Flow Through Intestine in Development of Irreversibility.* (23747)

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There is substantial evidence that bacterial endotoxins derived from the intestinal flora are the cause of irreversibility to transfusion and death in prolonged hemorrhagic shock(1-3). The accumulation of these toxins in the blood and tissues is in a large part owing to the failure of the systemic antibacterial and detoxifying defenses(4-10). Since the intestinal flora are the source of the endotoxins it is possible that the deficient flow through the intestine with resulting injury to the local defenses may also play a significant role in this phenomenon. Consequently, the effect of deficient flow through the intestine on the systemic circulation was investigated.

Method. A group of healthy adult New Zealand white rabbits (av wt 2-3 kg) were submitted to laparotomy under general or local anesthesia. The superior mesenteric artery was encircled by a silk thread, both ends of which were passed through a stiff plastic tube long enough to reach from the artery to a subcutaneous pocket, where the ends were buried. Two or 3 days later the ends of the loop were recovered under local anesthesia and the artery occluded by tightening them against the plastic tube. The artery was released after one hour, and the thread and tube removed without entering the abdomen. On several occasions the abdomen was opened to allow observation of the result of the occlusion, and it was noted that the occlusion produced spasm and pallor of the entire small intestine and the proximal half of the colon. The observations made included pulse rate, blood pressure (via a catheter in the femoral artery), rectal temperature, total and differential white blood count, hematocrit, plasma volume (by the radioactive iodinated albumin method, taking a single sample at 15-20 minutes), blood and peritoneal cultures,

survival time and gross pathology at post mortem examination. The experiment was repeated in 2 additional groups of rabbits, one of which (Group II) was pretreated with antibiotics, the other (Group III) with dibenamine. The antibiotics were 100 mg of Neomycin and 10,000 units of Bacitracin given in 10 ml saline by gavage for 4 days, the last dose 2-3 hours before occlusion of the artery. Dibenamine was given intravenously in a dose of 20 mg/kg. In the later experiments with this drug it was given 18 hours before occlusion, because in earlier experiments, in which the drug was given 3 hours before occlusion, nystagmus, ataxia and convulsive seizures occurred in several animals which died during occlusion or soon after release of the occlusion. By the use of the longer interval it was possible to exclude these complicating effects of the drug, which develop soon after its administration. In one additional group of rabbits the occlusion was not released, and no treatment was given. In this group only the gross response and survival time were observed.

Results. *Group I: Occlusion without pretreatment.* The course of events was consistently as follows: During the hour of occlusion there was no significant change in general appearance, alertness, or vital signs. Following release of the occlusion the animal was quiet and responded little when prodded. Respirations were rapid and shallow. Of 37 rabbits in this group 33 (89%) died within 40 minutes to seven hours (average 2 hours) after release of the occlusion. In 12 animals the blood pressure was observed continuously throughout the experiments. The mean systolic blood pressure before occlusion was 100 mm Hg (range 80-110), and during occlusion it was 84 (range 78-110). Immediately after release of the occlusion it fell to 76 (range 65-105), where it remained with slight fluctuations until about one hour before death, when

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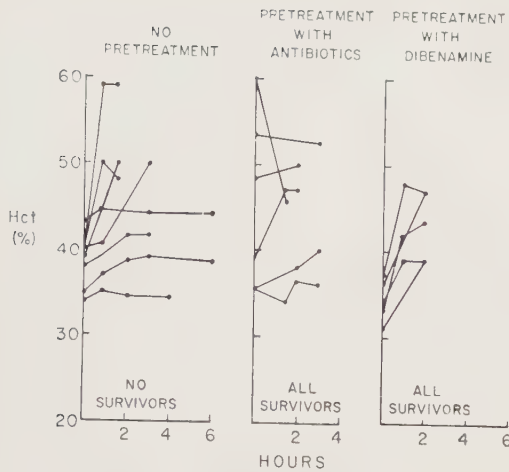


FIG. 1. Hematocrit values following release of one hr occlusion of superior mesenteric artery. The hematocrit curves for non-survivors pretreated with antibiotics or dibenamine were essentially the same as for survivors. In figure for dibenaminized animals values are given for 5 experiments. Terminal values were the same in 2 pairs of animals.

it fell abruptly to 30-50 mm Hg, with the manifestations of profound shock. Hematocrits were determined in 16 experiments. Measurements were made before occlusion and several times following release of the occlusion. In most instances, these did not change significantly (Fig. 1). In 8 experiments simultaneous plasma volume and hematocrit determinations were made before occlusion, and again one or more hours after release of the occlusion. In 6 instances the second plasma volume measurements were obtained while the systolic pressure was still at or above 65 mm Hg. In these the average plasma volume had fallen 18% (range 0-35%) (Fig. 2). In the remaining 2 the second plasma volume determination was made shortly before death, and the fall was 55% and 65% respectively. In most of the 8 experiments there was no correspondence between the change in hematocrit and the change in plasma volume.

During the occlusion there was a slight fall in the total white count, but no shift in the per cent of granulocytes. One hour after release of the occlusion there was a moderate leucopenia, and a severe granulocytopenia. The fall in granulocytes was from a mean control value of 67% of the total white cell

count to one of 22% (range 18-25%).

The rectal temperature declined steadily until death, reaching some 10° below the initial value in the longest survivors. At post mortem examination there was no urine in the bladder. The intestine showed localized and diffuse intramural hemorrhages, most numerous in the cecum, and patches of diffuse violaceous discoloration. There was a variable but small amount of edema in the terminal ileum. There was no bloody fluid in the gut lumen, no unusual amount of peritoneal fluid, and no peritoneal inflammatory reaction. Swab cultures of the peritoneum were sterile. So were all ante-mortem and post-mortem blood cultures.

To evaluate the importance of the measured plasma volume deficit in the development of shock, four rabbits were treated with repeated infusions of plasma, given so as to prevent a rise in hematocrit (*i.e.* 10 ml every 30 minutes for 6 hours), beginning immediately after the occlusion. Although the survival time was prolonged to 12, 22, 24 and 36 hours respectively, none survived, and at post mortem all 4 rabbits showed the same findings as those not so treated.

In contrast to an average survival time of 2 hours after temporary occlusion, rabbits

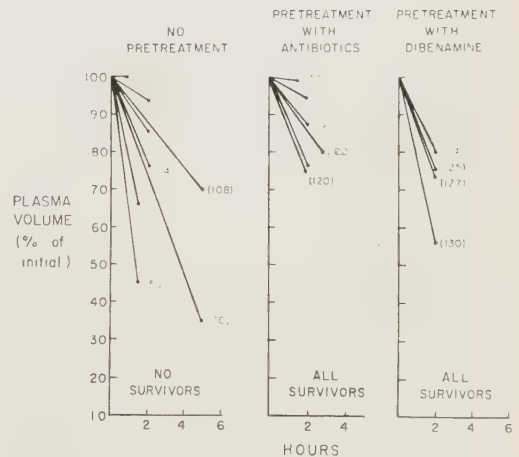


FIG. 2. Changes in plasma vol and hematocrit following release of one hr occlusion of superior mesenteric artery. Hematocrits are in parentheses adjacent to plasma vol measurements and expressed in % of preocclusion values. Values for non-survivors pretreated with antibiotics or dibenamine were essentially the same as for survivors.

with permanent occlusion remained in apparently good condition for 6 hours, and then developed severe hypotension followed by death an hour or so thereafter.

Group II: *Pretreatment with antibiotics.* Of 21 rabbits in this group 16 (76%) survived. The 5 which died lived longer than those in Group I, but the course of events and the post mortem findings were essentially the same as in Group I. The blood pressure was observed continuously in 6 experiments. The mean systolic blood pressure before occlusion was 97 (range 85-110). During occlusion it fell to 88 (range 80-100). Immediately after release of occlusion it fell to 63 (range 45-75), where it remained for the subsequent several hours of observation. In these 6 experiments plasma volume measurements were made before occlusion, and again about 2 hours after release of occlusion. The changes were about the same as those in Group I (Fig. 2). Simultaneous hematocrit determinations did not reflect these changes (Fig. 1 and 2).

The initial white blood count fell slightly during the occlusion, but the granulocyte count did not. Following release of the occlusion a granulocytopenia developed, but it was less severe than in the untreated group *i.e.* 43% (range 32-53%) of the total count as compared to 22% (range 18-25%), and began to return toward normal by the fifth hour.

The rectal temperature fell progressively as in the untreated group. Severe shock did not develop. By the sixth hour these animals were recovering, and the next day they appeared entirely well.

Nine rabbits, not included in the series, were prepared the same way, but were killed 4 hours after release of the occlusion. Of these two showed intramural intestinal hemorrhage, 3 showed slight scattered punctate hemorrhages, and 4 showed no gross changes whatever.

Group III: *Pretreatment with dibenamine.* Fifteen (75%) of 20 rabbits pretreated with dibenamine recovered completely. (Three of the five that died exhibited nystagmus, ataxia and convulsive seizures before and during the occlusion and may, therefore, have succumbed to the toxic effects of the dibenamine.) The blood pressure was observed continuously in 5

animals. The mean systolic pressure before occlusion was 88 (range 80-100). No change occurred during occlusion. After release of the occlusion 3 animals developed a severe hypotension (30-45 mm Hg) for the subsequent 3 hour period of observation. In the other 2 the systolic pressure fell only slightly and remained unchanged at 70 and 90 mm Hg respectively. All 5 animals were among the survivors. Plasma volume measurements were made in 4 experiments. The fall 2 hours after release of the occlusion was comparable to that in the other groups (Fig. 2). The changes in hematocrit were more proportionate to the changes in plasma volume in this group than in the others.

The white blood counts showed little decline, and granulocytopenia did not develop. The rectal temperature fell as in the other groups. In several experiments not included in the series the rabbits were killed 4-6 hours after release of the occlusion. None showed intestinal hemorrhage or edema. The peritoneum was normal. In the 5 rabbits that died the findings were the same.

Discussion. Our experiments were designed on the assumption that the nature of injury to the gut inflicted by 1 hour of total ischemia should be similar to that produced by 4 or more hours of deficient flow that results in failure of animal in hemorrhagic shock to respond to transfusion. The shock which developed soon after release of occlusion was not unlike the hemorrhagic shock which is irreversible to transfusion. For vascular collapse was profound, and was not corrected by plasma volume therapy. Although this treatment prolonged life considerably, one cannot consider hypovolemia a primary factor in causation of shock, or in its lethal outcome, because hypovolemia was about equal in all 3 groups of animals, and because recovery occurred in Groups II and III without plasma volume therapy.

That death following occlusion of the superior mesenteric artery was caused by absorption of endotoxins is indicated by the fact that non-absorbable antibiotics prevented shock and death in most animals. Presumably systemic defenses were intact until occlusion was released. The rapid collapse fol-

lowing release suggests flooding of circulation with toxin sufficient to overwhelm these defenses and to destroy the integrity of peripheral vessels, in much the same way as lethal intravenous dose of endotoxin to a healthy animal produces failure of peripheral circulation. The more rapid death in rabbits in which occlusion was released than in those in which it was not released may be merely a measure of difference in rate of transfer of toxin from the gut to the systemic circulation.

Since injury resulting from intestinal ischemia in a healthy animal can produce irreversible shock, it is permissible to infer that a similar less rapidly induced intestinal injury can occur in hemorrhagic shock; and, therefore, when there is failure to respond to transfusion, it may be in consequence of toxin entering the circulation from the gut (whether or not there is any other source of toxin) in an animal whose systemic defenses against toxins are already badly damaged.

Severe hypotension which occurred in some dibenaminized animals following release of occlusion, may be due to proportionately greater hypotensive effect of a given amount of hypovolemia in the dibenaminized animal, than in the non-dibenaminized animal(11). Since hypovolemia and hypotension were as severe in these animals as in untreated ones, and did not prevent recovery, the protective effect of dibenamine must be related to some other phenomenon. Data suggesting that it acts to block endotoxins are as follows: 1. Endotoxin in blood of hemorrhagic shock is prevented from injuring a susceptible test animal if the latter is dibenaminized shortly before the test(12). 2. The hemorrhagic lesion in the gut in hemorrhagic shock is due to endotoxin(2,10). This lesion was present in the non-dibenaminized animal following release of the occluded superior mesenteric artery, and absent in the dibenaminized animal subjected to the same procedure.

Thomas has demonstrated that endotoxin potentiates local action of adrenalin, and that dibenamine blocks the effect of endotoxin by its anti-adrenergic property(13). One may therefore explain the protective effect of dibenamine in animals with occlusion of superior mesenteric artery by the hypothesis that it

blocks the action of endotoxin rather than prevention of endotoxemia.

The foregoing analysis of the data is relevant to the observation that provision of an adequate flow of arterial blood to the liver (14), or the intestine of the animal(15) in severe and prolonged hemorrhagic shock by cross circulation with a healthy donor prevents development of irreversibility to transfusion and death. It is such phenomena as are under consideration here that may also be involved in the unexplained deaths following temporary interruption of flow through the lower thoracic aorta during aortic graft surgery, or shock and death that not infrequently follows surgical reduction of a strangulated hernia without resection of bowel with a marginal vascular integrity.

Summary and conclusions. 1) Transient ischemia of the gut in rabbits produced a type of shock that is similar to the state of hemorrhagic shock which is irreversible to transfusion. Since the shock was as profound and as lethal whether a substantial plasma volume loss did or did not occur, hypovolemia was not an essential feature. This is further demonstrated by the observation that plasma volume therapy delayed but did not prevent death, and by the observation that dibenaminized animals and animals given antibiotics developed an equal degree of hypovolemia, but survived without blood volume therapy. Bacterial cultures were sterile, and the only notable gross lesion at death was intramural hemorrhage in the gut wall. 2) Dibenamine given in advance of the occlusion prevented the development of irreversible shock and resulted in recovery without therapy. When killed before or after recovery from shock these rabbits did not show a visible injury to the gut. 3) Non-absorbable antibiotics given in advance achieved the same protection as dibenamine. 4) Dibenamine prevented and the antibiotics reduced the degree and duration of the granulocytopenia which occurs in untreated animals. 5) A fall in rectal temperature occurred in all rabbits exposed to the vascular occlusion of the gut whether they developed shock or not, and whether they survived or not. 6) Transient ischemia of the gut produces an endotoxemia derived from

the intrainestinal flora sufficient to overwhelm the normal detoxifying potential, and so induces irreversible shock. 7) These observations are in conformity with prior evidence that irreversibility to transfusion in hemorrhagic shock is due to endotoxins derived from the intestinal flora, and suggest further that deficient flow through the intestine accounts for the invasion of circulation by these endotoxins in sufficient quantity to produce irreversibility.

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Bactericidal Activity of Normal Serum Against Bacterial Cultures. I. Activity Against *Salmonella typhi* Strains. (23748)

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The bactericidal action of normal serum, long recognized as a factor in the total complex of "non-specific" resistance to infection, is still of uncertain significance. Smooth forms of the Gram-negative bacilli are generally relatively virulent and resistant to the bactericidal action of normal mammalian serum; rough forms are avirulent and more susceptible. On the other hand, it has been reported that *Escherichia coli* is more resistant to normal serum than pathogenic microorganisms of the Enterobacteriaceae(1). This report requires modification since it is now recognized that certain serotypes of *E. coli* may be pathogenic and that different strains of a bacterial species may show marked differences in their susceptibility to the bactericidal action of normal serum. Also, in the case of anthrax, the serum of the naturally immune dog has little effect against the anthrax bacil-

lus, but serum of the susceptible rabbit is bactericidal(2). Moreover, different mechanisms may be involved in serum bactericidal action. Serum substances, active against some Gram-positive organisms, are non-specific and heat stable. Complement is probably not involved in the activity of these substances(3). In contrast, the action exerted upon most Gram-negative organisms is mediated by natural antibody and complement and recently the properdin system has been implicated(4). Previous investigators have concluded that bactericidal antibodies in normal serum active against Gram-negative organisms are species specific(5,6); others have considered them non-specific or of limited specificity(7,8).

The present study was undertaken to reinvestigate some aspects of the bactericidal action of normal serum with the quantitative

spectrophotometric growth assay method(9). *S. typhi* strains of varying antigenic constituents were tested for their resistance to normal serum. In addition, the resistance of the strains to phagocytin(10), another substance implicated in natural defense mechanisms, and to chloramphenicol, the antibiotic of choice in typhoid fever therapy, was determined.

Materials and methods. S. typhi strains. Cultures were received from Mr. Arthur Abrams from the stock collection of this laboratory, and were maintained on meat extract agar. Strains Ty6S, ViI, Ty2, Watson, H9C1, and O901 have been described by Felix and Pitt(11). *S. typhi* strain 47 was received from Lt. Col. Oscar Felsenfeld. It was isolated recently from the holy water in the Temple of Karat in central Thailand where it was implicated in the etiology of a serious outbreak of typhoid fever. Strain 63 had been isolated posthumously from the meninges of a typhoid fever patient in 1935. Strain 58 had been isolated in Panama from the feces of a "chronic carrier" of long standing and is used for production of typhoid vaccine in the U.S. The antigenic constituents of various strains are given in Table I. *Sera.* The human serum pool was obtained from four individuals who had no history of typhoid fever or of immunization and whose sera lacked detectable *S. typhi* agglutinins; the rabbit serum was a pool from three animals; the guinea pig material was the lyophilized commercial product of Swanson Biological Laboratories and was reconstituted with distilled water to its original volume. Sera were stored at about -30°C ; all testing was performed within 3 weeks of drawing of the blood or of reconstitution of the lyophilized serum. *Bactericidal tests.* Tests for measuring bactericidal activity of normal serum were performed by a modification of the spectrophotometric growth assay technic developed for the titration of bactericidal antibody(9). In brief, the test involved a series of test tubes which contained variable amounts of the test serum, and duplicate control tubes without serum. Foreign complement was not added; the test serum itself served as the complement source. All tubes were brought

to constant volume with the saline diluent containing 0.85% NaCl and 0.063% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. The appropriate culture, prepared by suspension of the growth from an overnight slope culture (maintenance meat extract agar) in broth was optically standardized and then added in constant amounts (approximately 2×10^7 organisms) to each tube. The tubes were incubated in a water bath at 37°C for one hour when the bactericidal reaction occurred. Determination of the relative number of surviving organisms was then made by addition of 2.5 volumes of broth which terminated the reaction and practically eliminated any contribution of residual non-viable organisms to the final turbidity. When the set of tubes reached a suitable reading range with the control tubes still in the log-growth phase, the tubes were chilled in ice water to check further growth and the optical densities of the tubes were read with a broth blank in a Coleman Universal Spectrophotometer. The percentages of growth, which approximate the percentage survival of the organisms, were obtained by dividing the optical density for each experimental tube by the average of the optical densities of the control tubes, and multiplying by 100. Linear representations of the data were obtained by plotting the logarithm of the serum amount against the probit of the percentage growth. From the line, a 50% endpoint by interpolation, or if required, by extrapolation, and the slope of the response line was estimated. The bactericidal action of phagocytin prepared as outlined in reference 10 and of chloramphenicol were also determined similarly with the substitution of graded amounts of these substances in place of serum. The diluent for the tests with phagocytin was the intracellular salt solution proposed for use with that substance(10). *Agglutination tests.* These tests were performed according to the procedures given by Felix and Pitt(11). *Vi content of the strains.* HCl extracts of the acetone-dried organisms (3% of the dried organisms in 1% concentrated HCl) were prepared(12). The highest dilution or smallest amount of the neutralized extract required to inhibit the agglutination of maximally sensitized sheep red blood cells coated with purified Vi antigen(13), obtained

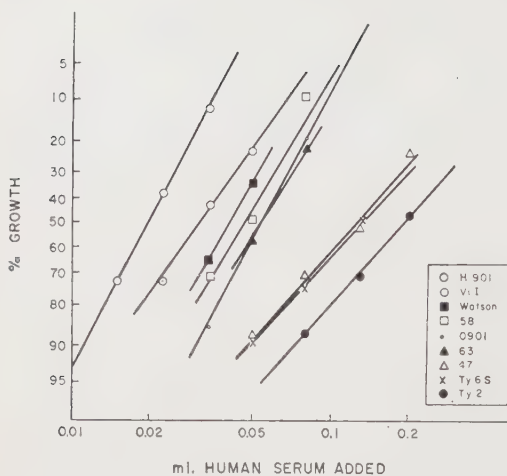


FIG. 1. Growth inhibition of *S. typhi* strains by human serum.

from Dr. Marion Webster, by an anti-Vi (*Paracolobactrum ballerup*) rabbit serum was determined.

Results. Bactericidal tests. The results obtained in testing the bactericidal activity of normal sera against the *S. typhi* strains are given in Fig. 1, 2, 3. Generally the strains were considerably more susceptible to human serum than to guinea pig or rabbit sera. Strains Ty2 and 47 were the most resistant to the bactericidal action of the normal serum of all 3 species. Strain 6S, rather resistant to human and rabbit sera, was more susceptible to guinea pig serum. This unexpected species difference was confirmed with an additional pool of guinea pig serum. At the other extreme, strain H901 was the most susceptible to the bactericidal action of the sera of all species. The other strains intermediate in their order of activity varied slightly in their relative susceptibility to the sera of the 3 species. In addition, the slope response of the bactericidal reaction was low where the 50% end-points of the serum were high and conversely the slopes were high when the 50% end-points of the serum were low. *O-inagglutinability.* *O-inagglutinability* of the strains was determined by comparison of their agglutinability in the killed and living state by anti-O (strain O901) rabbit serum. The results (Table II) indicate that the *O-inagglutinability* of the strains containing both O and Vi antigens, in descending order, is as follows:

Ty2, 47, 63, Watson and 58. **Vi content.** The results of the hemagglutination-inhibition tests with the extracts of the different strains are given in Table II. The procedure was found to be sensitive to about 2 $\mu\text{g}/\text{ml}$ of purified Vi substance. Calculation indicated that the content of Vi substance in strain Ty2 was about 0.4% if complete extraction were assumed. For strains 47 and ViI, it would be approximately half that value. The results have indicated that the Vi content and *O-inagglutinability* in strains possessing both antigens show a marked correlation. The living cultures were also tested against an anti-Vi (*P. ballerup*) rabbit serum. Strains that show the strongest agglutination with this serum are those poorest in Vi antigen. Decreased agglutinability by increased amounts of Vi antigen has been observed also by Felix and Pitt(11). The agglutination of living cultures of strains H901 and O901 by anti-Vi (*P. ballerup*) was unexpected and may be attributed to the presence of antigenic factors of those organisms, other than Vi, cross-reactive with *P. ballerup*.

Incubation of strain Ty2 cultures at temperatures other than 37°C. Nicolle, Jude, and Diverneau(14) reported that *S. typhi* grown at 18°C or 41.5°C fails to produce Vi antigen and is immune to Vi phage. At these temperatures, enzymes responsible for synthesis of

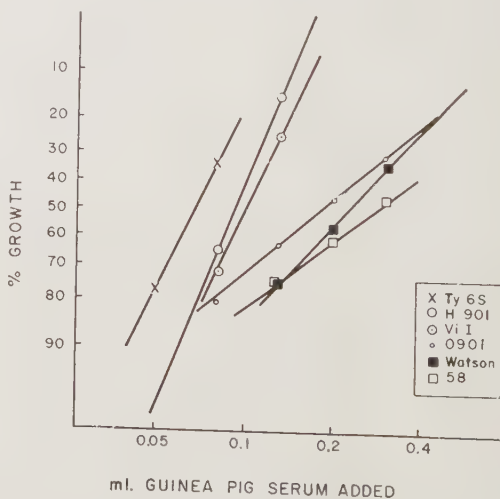


FIG. 2. Growth inhibition of *S. typhi* strains by guinea pig serum. Strains Ty2, 47, and 63 were too resistant for titration (50% point greater than .3 ml of serum).

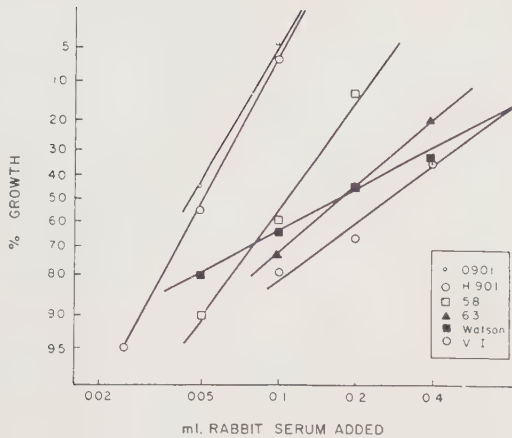


FIG. 3. Growth inhibition of *S. typhi* strains by rabbit serum. Strains Ty2, 47, and 6S were too resistant for titration (50% point greater than .4 ml of serum).

Vi antigen exist in the cells for a number of generations despite the absence of the antigen. Three cultures of strain Ty2 were accordingly grown on meat extract agar at 3 different temperatures: one at 41.5°C for 16 hours, another at 18°C for almost 3 days to provide adequate growth, and a third at 37°C for 16 hours. The living cultures were then tested simultaneously for agglutinability in anti-O and for susceptibility to the bactericidal action of normal serum; the acetone dried cells were tested for their Vi content by the hemagglutination-inhibition procedure. The results of these tests (Table III) indicate that the partial loss of Vi antigen by growth at temperatures above or below 37°C, reflected by a 4-fold drop in hemagglutination-inhibition, was accompanied by a 5-fold increase in susceptibility to the bactericidal action of normal human serum. However, the concomitant 16-

TABLE I. Antigens Present in *S. typhi* Strains.

| Strain | Antigens | | |
|--------|----------|----|----|
| | Vi | O | H |
| Ty6S* | + | — | tr |
| ViI* | + | tr | tr |
| Ty2 | + | + | + |
| 47 | + | + | + |
| 63 | + | + | + |
| Watson | + | + | + |
| 58 | + | + | + |
| H901 | — | + | + |
| O901 | — | + | — |

tr = trace amt; + = present; — = absent.

* Strains serologically rough.

fold loss in O-inagglutinability in the 41.5°C culture and the 64-fold loss in the 18°C culture indicates the marked sensitivity of O-inagglutinability as an index of the Vi content of a culture. Reincubation of the cultures at 37°C for 16 hours resulted in almost complete restoration of O-inagglutinability and resistance to normal serum.

Specificity of bactericidal action of normal serum. A normal human serum was divided into 3 aliquots; one of these was absorbed at 4°C with saline washed heat-killed organisms of strain O901, another aliquot was absorbed with organisms of strain Ty6S, and a third aliquot was untreated and served as a control. The aliquot absorbed with strain O901 showed a 4-fold loss of bactericidal activity against

TABLE II. O and Vi Antigenic Analysis of *S. typhi* Strains.

| Test strain | Anti-O titers* | | Vi hemagglutination inhibition titer† | Anti-Vi titer* (living cultures) |
|-------------|-----------------|-----------------|---------------------------------------|----------------------------------|
| | Living cultures | Killed cultures | | |
| Ty6S | Neg. at 1/20 | Neg. at 1/20 | 128 or greater | 40 |
| ViI | " | " | 16 | 80 |
| Ty2 | 20 | 5120 | 32 | 80 |
| 47 | 80 | " | 16 | 320 |
| 63 | 160 | " | 8 | 320 |
| Watson | 640 | " | 8 | 640 |
| 58 | 1280 | " | 4 | 1280 |
| H901 | 2560 | 2560 | No inhibition | 160 |
| O901 | " | " | " | 80 |

* Titer represents reciprocal of highest reacting dilution.

† Titer represents highest dilution inhibiting agglutination.

strains V58, Ty2, and O901, which contain all the *S. typhi* O antigens, an approximate 2-fold loss in activity against *Salmonella paratyphi* A and *Salmonella strasbourg*, organisms which possess one of the O antigens of *S. typhi*, and no loss against *S. typhi* Ty6S, *Salmonella newington*, and *Salmonella oranienburg*, organisms which do not possess the *S. typhi* O antigens. On the other hand, absorption of normal serum with unwashed organisms of strain Ty6S which retained its Vi antibody combining capacity effected an 8-fold loss of activity against the homologous strain and

TABLE III. O-inagglutinability, Relative Vi Content of Extracts, and Resistance to Bactericidal Action of Normal Human Serum of Strain Ty2 Cultivated at 3 Different Temperatures. These cultures were later all reincubated overnight at 37°C and retested.

| Temp of growth, °C | Anti-O titer with living cultures | Hemagglutination inhibition of extracts in Vi system | Bactericidal tests | |
|----------------------------|-----------------------------------|--|---|-------|
| | | | ml normal human serum for 50% end-point | Slope |
| 37 | 40 | 32 | .24 | 2.7 |
| 18 | 2560 | 8 | .047 | 3.8 |
| 41.5 | 640 | 8 | .046 | 3.6 |
| After reincubation at 37°C | | | | |
| 37 | 40 | Not done | .26 | 3.0 |
| 18 | 80 | " | .15 | 2.5 |
| 41.5 | 80 | " | .15 | 3.1 |

ViI, but no loss with strains Ty2, 58 and O901.

Phagocytin. The bactericidal activity of phagocytin was determined against the *S. typhi* strains (Table IV). Strains Ty2, 47, and 6S, the most resistant to normal serum constituents, were among the most susceptible to phagocytin. *Chloramphenicol.* The typhoid strains were tested against varying amounts of antibiotic in saline solution and 50% endpoints were obtained (Table IV). The relatively uniform resistance of the strains to chloramphenicol (mean of the 50% endpoints, 1.05 µg, with average deviation equal to 0.17 µg) is in contrast to their greater variability to phagocytin (mean of 0.033 ml with average deviation equal to 0.013) and to normal human serum (mean 0.078 ml with average deviation equal to 0.048).

Discussion. Although typhoid fever is a human disease, normal human serum possesses considerable greater bactericidal activity against *S. typhi* than does rabbit or guinea pig serum. Obviously the resistance of a species to a particular parasite cannot be defined simply by the bactericidal action of its serum. Strain Ty2, of maximum mouse virulence and of proven virulence for chimpanzees(15) was the most resistant of all strains tested, followed by strain 47, responsible for a recent severe human outbreak. Generally, resistance to normal serum was positively correlated with the Vi content and O-inagglutinability of the

strains. The experiments in which strain Ty2 was cultivated at temperatures other than 37°C with loss of resistance to normal serum also demonstrated this association. An abnormal temperature, however, may not be of advantage to the host. Pasteur found that chilling reduced the resistance of birds to anthrax. Subnormal body temperatures also lower the resistance of rabbits to *Treponema pallidum*. The relatively high resistance of strain 6S to rabbit and human serum was another interesting finding. The large amount of Vi antigen in this strain may prevent access of serum substances to the susceptible R antigen of the cell(16). Also noteworthy was the relatively greater susceptibility of strain H901, compared to O901, a variant of H901 lacking the H antigen. This finding supports the belief that the H antigen plays no part in the virulence of *S. typhi*. In addition to the endpoints of the response, the slopes point to a greater protective effect against a strain such as 58 compared to Ty2. Greater slopes were obtained against those strains requiring smaller amounts of serum for a 50% endpoint (Fig. 1,2,3). If the slopes are relatively low, it may be difficult to attain killing as high as 95% with the amount of available serum, although 50% endpoints are within reach. The significance of this point depends upon whether almost all of the organisms must be removed by the bactericidal reaction for effective resistance to infection.

The absorption experiments were concerned with the question of the specificity of natural bactericidal antibodies. Our findings have

TABLE IV. Bactericidal Action of Phagocytin and Chloramphenicol against *S. typhi* Strains.

| Strain | Phagocytin | | Chloramphenicol | |
|--------|-------------------|---------------------|-------------------|---------------------|
| | 50% end-point, ml | Order of resistance | 50% end-point, µg | Order of resistance |
| 6S | .017 | 8 | .9 | 9 |
| ViI | .019 | 7 | 1.3 | 3.5 |
| Ty2 | .034 | 6 | .98 | 8 |
| 47 | .04 | 4 | 1.4 | 1.5 |
| 63 | .044 | 2.5 | 1.3 | 3.5 |
| Watson | .050 | 1 | 1.0 | 7 |
| 58 | .044 | 2.5 | 1.4 | 1.5 |
| H901 | .011 | 9 | 1.1 | 5.5 |
| O901 | .040 | 5 | 1.1 | 5.5 |

indicated a marked specificity. Absorption with one organism resulted in a loss of activity only against other bacterial species which possess a cross-reactive antigen. Our results suggest that the natural bactericidal antibodies active against strains Ty2, 58, and O901 are specific for the O antigen since absorption of serum with strain O901 organisms resulted in almost complete loss of activity against those strains. Absorption with the Vi rich, but serologically rough strain Ty6S resulted in a loss of activity against the homologous strain and the semirough strain, ViI, but not against Vi antibody susceptible strains, Ty2 and V58(16). The results imply that the R antigen, in addition to the O antigen, is the susceptible target for bactericidal activity of normal serum against strains containing these antigens. Natural antibodies specifically oriented against the Vi antigen are either not present or their concentration is too low to exert a bactericidal effect. The relationship of these findings to the bactericidal action of the nonspecific properdin system(4) is of interest. Our results indicate that natural bactericidal antibody of marked specificity plays the major role. A recent report has indicated also that the properdin content of normal animal sera of different species did not correlate with differences in the bactericidal activity of the blood of the animals against *Pasteurella tularensis*(17). That the natural antibodies are specific does not necessarily favor any particular theory concerning their origin. It merely means that they react with certain organisms, and not with others, because of a chemical correspondence of certain specific combining groups.

It was considered of interest to determine relative resistance of the typhoid strains to phagocytin, an extract of rabbit polymorphonuclear leucocytes, since phagocytin may constitute another factor in natural resistance. No correlation was shown to exist between the resistance of the various strains to phagocytin and to normal serum. Strains Ty2 and 47 were not particularly resistant to phagocytin, and one is tempted to believe that serum may be of greater significance than phagocy-

tin in protection against typhoid organisms. In contrast to the variability of the strains to the bactericidal action of serum and phagocytin, the uniform resistance of the different strains to chloramphenicol indicates that the action of the antibiotic is directed against a process or target, possibly protein synthesis (18), common to all the strains. It is not too unexpected that, as the data suggest, the bactericidal actions of normal serum, phagocytin, and chloramphenicol involve different mechanisms.

Summary. The resistance of strains of *S. typhi* to the bactericidal action of normal guinea pig, rabbit, and human serum was determined. The order of resistance of the strains to sera of different species was in close agreement, but human sera exerted a greater bactericidal effect than rabbit or guinea pig sera. The resistance of the strains containing both O and Vi antigens was associated with the O-inagglutinability or Vi content of the organisms. The natural bactericidal antibodies against *S. typhi* were of marked specificity and directed against either the O or R antigens. The resistance of the strains to phagocytin or to chloramphenicol did not correlate with their resistance to normal serum components.

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Studies on *in vivo* Stability of an Iron Chelate. (23749)

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N-hydroxyethylethylenediamine triacetic acid is a chelating agent similar to ethylenediamine tetraacetic acid, but with one acetic acid group replaced by an ethanol group. This alters the chelating properties for iron and other heavy metals(1). This compound has been studied in hemochromatosis and found to be effective in the removal of iron from the tissues when given intravenously(2). Since the stability constant of the iron chelate formed *in vitro* is high, it might be expected to hold iron firmly in the body. However, experimental studies have shown that when ferric chelate is injected intramuscularly in iron-deficient mice, hemoglobin regeneration occurs, and degree of hemoglobin response is slightly greater than with the corresponding chelate of ethylenediamine tetraacetic acid.[†] The studies here reported were carried out in 2 iron deficient males. One received Fe⁵⁹ tagged ferric disodium N-hydroxyethylethylenediamine triacetate intramuscularly, and the second received the tagged chelate orally, and by duodenal tube.

Methods. The Fe⁵⁹ tagged ferric chelate of N-hydroxyethylethylenediamine triacetic acid was prepared from the disodium salt of this chelating agent by simultaneous addition of a ferric chloride solution and dilute sodium hydroxide within a pH range of 6.0-7.5. This preparation was evaporated to a small volume and sterile filtered, resulting in a solution stable for 3 weeks. The first subject, T.R., a

71-year-old male, showed depletion of body iron stores with hypochromic anemia after repeated phlebotomies for study purposes. At the time of the study, fasting serum iron levels varied between 19 and 24 $\mu\text{g}\%$ with unsaturated iron binding capacity (UIBC) between 262 and 286. Hemoglobin values varied between 9.4 and 9.7 g, hematocrit between 32 and 35%, RBC between 4.14 and 4.33 million, reticulocytes between 0.1 and 1.6%, and indices showed MCV 75-85, MCH 22-23, MCHC 27-29. He was given 2.75 ml of iron chelate by intramuscular injection in the left hip, representing 50 mg elemental iron and 10 μC Fe⁵⁹. Surface counting was carried out over the injection site and other body areas at regular intervals with a scintillation detector. Blood and plasma samples were analyzed for radioactivity in a "well-type" scintillation detector. Fractional urine collections were obtained on the day of injection and at daily intervals thereafter. These were evaporated to a small volume before counting in three 4 ml aliquots. Feces collected over a 4 day period were passed directly into a Waring Blendor, blending was carried out for 20 minutes with a small amount of water, and three 4 ml aliquots were taken directly from this slurry to be counted in the same "well" counter. After 12 days, studies were undertaken to determine the effect of therapeutic doses of iron chelate without radioactive iron on the serum iron and UIBC levels within the hours immediately following injection. Serum iron and UIBC values were obtained by a modification of the method of Schade(3) using Nitroso R as the

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[†] Rubin, M., personal communication.

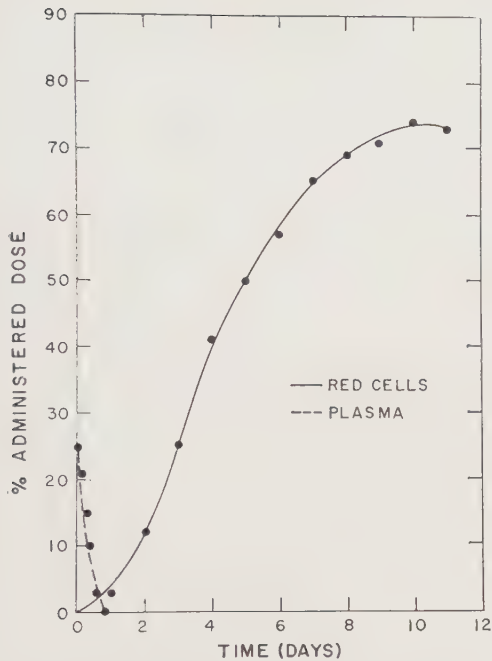


FIG. 1. Disappearance of Iron⁵⁹ from plasma and its incorporation into circulating red cells.

ferroin reagent. J.E., a 19-year-old male, also showed iron deficiency with hypochromic anemia after repeated phlebotomies for study purposes. One month prior to this study, his fasting serum iron level was 36 $\mu\text{g}\%$ with UIBC of 170. At the time of the study, the fasting serum iron was 22 $\mu\text{g}\%$ with UIBC of 280, the hemoglobin 8.6 g, hematocrit 28%, RBC 3.6 million, reticulocyte count 0.3%, and indices of MCV 77, MCH 24, and MCHC 30. While in a fasting state, he was given 25 ml of Fe⁵⁹ tagged ferric chelate intraduodenally per Rehfuess tube, followed by 75 ml water. This dose was equivalent to 50 mg elemental iron and 10 μC Fe⁵⁹. On the 12th day, this subject was fasted again and given 50 ml of the Fe⁵⁹ tagged ferric chelate orally, followed by 50 ml water and 50 ml tomato juice. This dose represented twice the iron content of the intraduodenal dose, or 100 mg elemental iron and 20 μC Fe⁵⁹. Hematologic values at the time of this study revealed a hemoglobin of 8.6 g, hematocrit 30%, RBC 3.66 million, and indices of MCV 81, MCH 23, and MCHC 28. Blood, serum, urine, and stool specimens were collected and counted in a manner similar to that for the

first subject.

Results. Surface counting at selected sites after intramuscular injection of 10 μC Fe⁵⁹ as the iron chelate showed that disappearance from the injection site (left hip) was rapid, with little radioactivity detectable 15 minutes later. Counting at this site after 2 to 4 hours was stable and paralleled that over the liver. On the 7th day, counts over both hips were similar, further suggesting that there had been complete uptake from the injection site.

Fig. 1 shows radioactivity of the plasma and incorporation into the RBC of this subject. Maximal uptake by plasma occurred prior to the first collection, drawn 2 hours after injection. There was a gradual loss to the tissues and marrow, and, at 12 to 24 hours the uptake of Fe⁵⁹ by circulating RBC became apparent. Uptake by the RBC was rapid until the 4th to 5th day and reached maximal incorporation at about the 11th to 12th day. Based on the known hematocrit and a blood volume estimated 74 ml/kg, about 73% was present in the circulating RBC after 11 days.

Table I summarizes urinary and stool output of radioactivity in this subject. Little activity was found in the stool over a 4-day period. Most of the excreted Fe⁵⁹ was found in the urine within 5 hours, and this approximated the total amount excreted by both routes over 4 days, or 9% of the injected dose. This amount plus the 73% found in circulating RBC accounted for 82% of the injected dose. The remainder probably was retained in body tissues.

TABLE I. Summary of Urinary and Fecal Output of Radioactivity in Patient T.R. following Intramuse. Injection.

| Period, hr | Counts/min. | % inj. dose |
|---------------------|-------------|-------------|
| <i>Urine</i> | | |
| 0-5 | 602,400 | 8.1 |
| 5-12 | 53,404 | .7 |
| 12-24 | 1,125 | |
| 24-48 | 818 | |
| 48-72 | 275 | |
| 72-96 | 627 | |
| Total 4 days | 658,649 | 8.9 |
| <i>Feces</i> | | |
| 4 " | 13,016 | .1 |
| Total urine & feces | 671,665 | 9.0 |

TABLE II. Serum Iron and UIBC Levels following Intramusc. Injection of 40 mg Iron in Chelated Form.

| Time following inj., hr | Serum Fe, $\mu\text{g } \%$ | UIBC, $\mu\text{g } \%$ |
|-------------------------|-----------------------------|-------------------------|
| 0 | 22 | 248 |
| $\frac{1}{2}$ | 71 | 228 |
| 1 | 92 | 182 |
| 2 | 146 | 112 |
| 4 | 294 | 0 |
| 8 | 267 | 0 |

Since the uptake of this iron chelate was rapid from the intramuscular site, it was of considerable interest to determine the effect on the serum iron levels and UIBC. Table II records these results at various intervals after injection of 40 mg iron as the ferric chelate. Iron determinations by the method employed did not determine iron present in the serum in the chelated form.

Intraduodenal administration of the Fe^{59} tagged chelate in the second subject resulted in little uptake by plasma and RBC. Following administration of 10 μc minimal radioactivity was detected in plasma only at 8 hours, and incorporation into RBC of the small amount absorbed from the duodenum was not obvious until the 6th day. Seventy-two percent of the dose was detected in the 4-day stool collection while only 0.1% was found in the urine over 2 days. From the hematocrit determination and a blood volume estimated at 69 ml/kg, it can be calculated that 2.4% was present in the circulating RBC after 12 days. Twenty-five percent of the administered dose was unaccounted for, but may have remained in the gastrointestinal tract beyond 4 days. Oral administration of a tagged dose twice as large, representing 100 mg iron and 20 μc Fe^{59} , produced similar results. On the 9th day, RBC radioactivity represented 3.4% of the administered dose. Seventy-two percent again was found in the 4-day stool collection and 0.1% in the urine over 3 days.

Discussion. Uptake of the iron chelate from the injection site was rapid, indicating a rapid diffusion into circulating fluids. In the first subject, *in vivo* counting over both buttocks yielded similar results after 7 days, suggesting a complete uptake by that time. Unchelated iron was found in the serum $\frac{1}{2}$ hour after injection and completely saturated the

iron binding protein in serum by 4 hours, indicating that chelate breakdown was rapid after its entrance into the circulation. The clinical significance is that iron was available to the marrow within minutes after injection. Incorporation into circulating RBC, undoubtedly reticulocytes, first became obvious at 24 hours, indicating that the available iron was quickly utilized in hemoglobin formation.

Administration of the chelate by oral and intraduodenal routes was not effective. The incorporation of Fe^{59} into circulating RBC indicated an uptake from the gastrointestinal tract similar to that found with administration of ferrous sulfate in normal and iron deficient patients(4), suggesting that in each event the chelate was broken down in the gastrointestinal tract to form an inorganic iron salt. Our subject did not show color changes in his feces, but this might not be expected with the small dose given.

If the intramuscular use of a specific iron chelate is a means of introducing iron into the body, this method may have application in parenteral iron therapy. The intramuscular use of a chelate may also be applicable in other trace metal deficiency states and in the administration of radioactive elements for diagnostic and therapeutic purposes.

Summary. The Fe^{59} tagged ferric chelate of disodium N-hydroxyethylethylenediamine triacetate was administered to 2 subjects made iron-deficient by repeated phlebotomies. Intramuscular injection in one subject resulted in a rapid uptake from the injection site, early incorporation into circulating RBC, and effective utilization of Fe^{59} by the RBC. Oral and intraduodenal administration of the tagged chelate to the second subject showed that iron in this form was not readily absorbed from the gastrointestinal tract.

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Extraembryonic Vascular Deterioration in Irradiated Chick Embryo.* (23750)

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Extensive vascular damage in 6-day chick embryos has been observed following X-irradiation(1). Death within a week following median lethal doses of radiation received by 8 to 12-day-old embryos was characterized by hemorrhage and petechiae in the yolk sac and viscera(2). Extravasation of blood into the extraembryonic coelom of 4-day embryos was observed beginning $3\frac{1}{2}$ hours after exposure to 1200-20,000 r(3). The relative sensitivity of the chick embryo to radiation induced hemorrhage has recently been studied in terms of total dose, dose rate and stage of embryonic development(4). Hemorrhage was slight in 6-day embryos within a few hours following 600 and 800 r of 250 KVP X-irradiation. Twelve-day embryos, however, showed moderate hemorrhage following 600 r and extensive hemorrhage following 800 r (LD 95). The present work was undertaken as an attempt to quantitate the effects of X-irradiation upon the extraembryonic circulation of chick embryos.

Methods and materials. Twelve-day-old embryonated eggs were chosen because of the convenient size of the embryo, the extent of development of the extraembryonic circulation, and the marked hemorrhagic state which has been observed within a few hours after irradiation. A simple windowing procedure was devised which permitted rapid preparation of the embryonated eggs. A medially oriented 22 mm circular segment of shell was gently removed from the egg after being scored by a high speed motor-driven thin abrasive disc. The exposed shell membrane was then carefully removed without disturbing the underlying membranes. The shell aperture was then sealed with a circular silicone-grease rimmed microscope cover slip.

* Work performed under contract between Atomic Energy Commission and University of Rochester, administered by Dept. of Radiation Biology, School of Medicine and Dentistry.

After the fenestrating process, the eggs were returned to the incubator for 24 hours. They were inspected under low power magnification just before irradiation, and any which appeared damaged were rejected. The remainder were coded and randomly divided into irradiation and control groups. The irradiation was delivered to the eggs supported on wire mesh in a circulating air incubator through a cover of one-sixteenth inch Plexiglass. The eggs were maintained at $38 \pm 1^\circ$ C throughout the experiment. The 800 r were delivered at 17 r/min. from a 250 kvp Picker Industrial generator utilizing the following factors: 250 kvp, 15 ma, Al. parabolic + $\frac{1}{2}$ mm Cu filter; HVL 2.25 mm Cu; 71 cm target to center of eggs.

Microscopic observations of the extent of injury to the allantoic and vitelline vessels were graded on an arbitrary scale of 5 ranks. Zero indicated no damage or visible impairment of function, while increasing injury was recorded by unit increments to 4, which represented maximum injury, loss of function or death. The condition of the major and minor blood vessels, the capillaries and the status of the blood flow in these 3 classes of vessels were scored. The eggs were graded under 20 magnifications with the window momentarily removed. Each egg was examined by 2 independent observers at $2\frac{1}{2}$, 4, 7, and 21 hours following irradiation. To preserve objectivity during the scoring period, the observers were not told whether the eggs were from the control or irradiated groups. The scores by experienced observers usually agreed within a half unit for any particular observation. Representative specimens were photomicrographed at 25 magnifications by reflected light during the course of the experiment.

Results. Vascular injury was detectable within a few minutes following irradiation. The course of injury as it develops between $2\frac{1}{2}$ and 21 hours following irradiation is shown in Fig. 1. The apparent lessening of

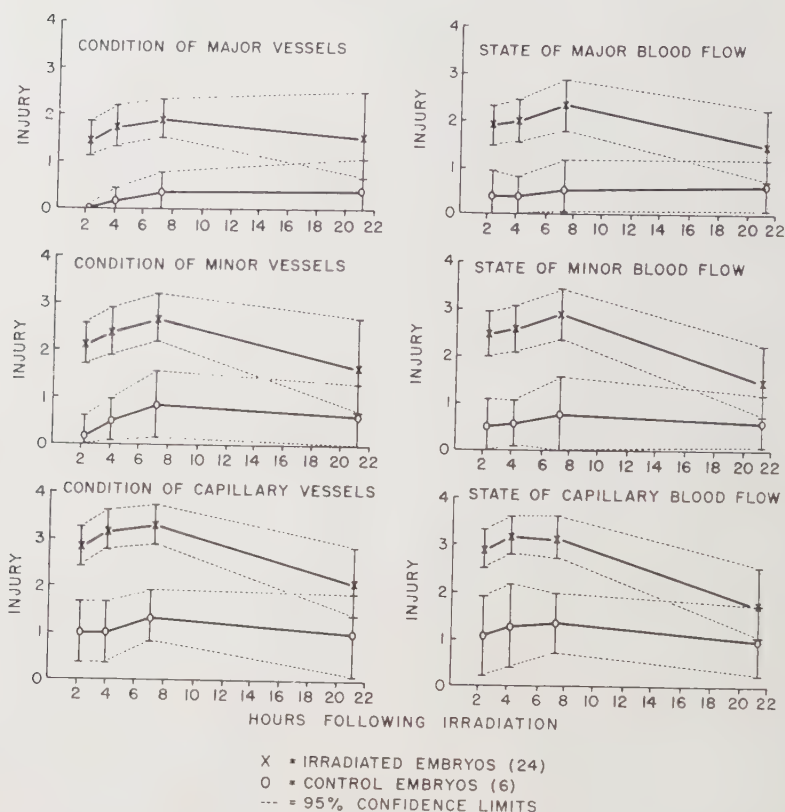


FIG. 1. Radiation injury to extraembryonic blood vessels of 12-day chick embryos.

damage after 7 hours is due to the procedure of recording only observations from surviving embryos. Non-survivors were recorded as 4 just prior to death after which the embryo was removed from the experiment (thus lowering the average value of subsequent observations on survivors, and decreasing the number of embryos in the group). By 21 hours 62% of the irradiated embryos were dead. None survived beyond 48 hours.

Injury was most pronounced in the capillaries. Extraembryonic vascular injury was observed simultaneously with the appearance of hemorrhage into the embryo. Blood flow through the vessels slackened and became sluggish in a sequence progressing from small to larger-sized vessels. By 3 hours even the largest vessels were affected. The general syndrome, as observed, was initiated first by a decrease in the blood flow, followed immediately by a constriction of vessel lumen. The vessels appeared to lose about half of their internal volume as the blood flow ceased.

Within an hour or two such vessels ruptured at several points of maximum constriction suggesting possible endothelial autolysis. Extravasated blood was observed in these regions. These observations suggested a weakening or defect of the vessel rather than a rupture resultant from normal pressure within the vessel. When many of the major vessels were affected, the embryo died. The time course varied from embryo to embryo but followed the above pattern until death. Each series of observations was completed within 30 minutes and recorded at the mean of the observation period. The prominent feature of these observations was the constriction and rupture of the extraembryonic vessels prior to the appearance of extravasated blood in that region. A possible contributing factor to the injury described for the extraembryonic vessels could have been the hemorrhage which has been noted within the embryo. These factors are subject to further investigation.

The condition of the blood vessels in non-

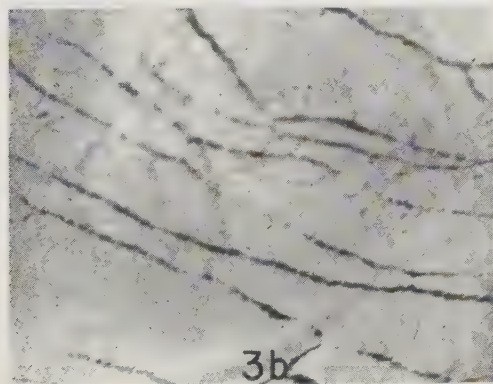
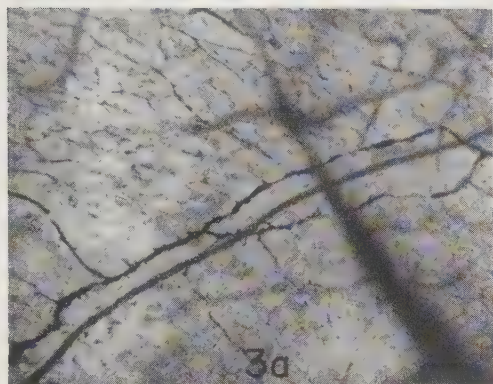
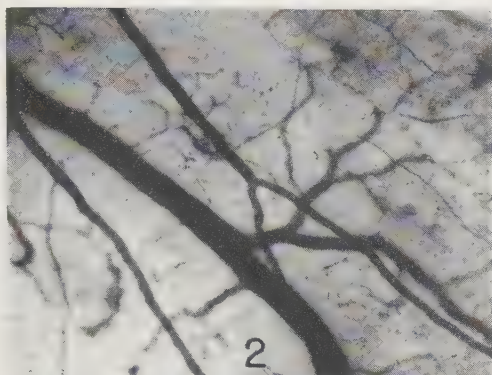


FIG. 2 and 3. Condition of extraembryonic blood vessels following 800 r. Fig. 2 = control. Fig. 3a = moderate deterioration 2.5 hr after irradiation. Fig. 3b = advanced deterioration 4 hr after irradiation. (15 magnifications.)

irradiated eggs is illustrated in Fig. 2. Capillary blood flow remained smooth and rapid throughout the experiment. Fig. 3a illustrates constriction and rupture of minor blood vessels at a time when many of the major vessels were still functional ($2\frac{1}{2}$ hours). Complete dissolution of the capillary network and rupture of many of the larger vessels prior to death of the embryo are illustrated in Fig. 3b (4 hours).[†]

Summary. Fenestrated eggs of 12-day chick embryos were given 800 r of 250 kvp X-rays. Injury to blood vessels of the extraembryonic membranes was detectable within minutes following irradiation. Deterioration was most rapid and extensive in the capillaries and least marked in the major blood vessels. Vascular disruption preceded the appearance of extravasated blood in that region. Intra-embryonic hemorrhage was also noted. Following irradiation the mortality was 62% at 21 hours and 100% by 48 hours.

[†] These observations have been repeated several times as teaching and student experiments with substantially the same results.

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Independent Differentiation in Components of the Pituitary Complex in the Wood Frog.* (23751)

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Blount(1) reported that the epithelial pituitary primordium in amblystoma does not differentiate after transplantation unless some brain tissue is transplanted with it. This concept of dependence of the buccal pituitary upon nervous tissue in differentiation has been supported by numerous investigators working with a variety of species and technics(2-9). Although these authors differ among themselves as to the degree of dependence of the pars anterior, they agree in finding that the pars intermedia does not differentiate nor does it secrete its pigment-regulating hormone if the buccal primordium is separated from its normal contact with the brain. Most investigators indicate that the infundibulum is the specific area of the brain which has this inductive influence over the pituitary primordium although Eakin(7) who, with his collaborators has explored this problem with the most varied approaches, has concluded that other neural tissue may substitute adequately for infundibulum.

On the other hand, working with various species of *Rana* not used by the other workers, Atwell and the present author have published several studies since 1935 in which independent differentiation of the isolated primordium into both pars anterior and intermedia was secured (for literature see 10,11). In Etkin's studies the intermedia showed great overgrowth and hyperfunction when transplanted heterotopically and he has provided other evidence(12) that the secretory activity of the pars intermedia is controlled by inhibitory influences from the hypothalamus. The differences in results were considered by various authors in the early 1940's to arise chiefly from the use of slightly more advanced primordia by Atwell and Etkin than by the other workers and perhaps also because of

some species differences in time of embryonic determination (see discussion in Blount(3)). The general picture which emerged at that time was that the hypothalamus has a stimulating or inducing influence on the differentiation of the pars intermedia in the embryo. Subsequently in the definitive tadpole and frog, this influence is replaced by an inhibitory control over growth and function of this lobe by the brain (Etkin(12)).

The present investigation is part of an attempt to ascertain at what time the presumptive pituitary becomes embryonically determined to form pars intermedia. This involves the verification of the independent differentiation of the definitive primordium in *Ranidae* by experiments with more precisely defined stages and by more critical evaluation of the absence of neural tissue in the graft than was attempted in any previously reported work. A separate question is that of the differentiation of the pars nervosa when the epithelial primordium is removed. Smith(13) in his classic study of hypophysectomy in the frog reported that the pars nervosa is reduced and abnormal in development in such animals. Our present concept of the nervosa as essentially a storage organ for neurosecretion(14) raises the question as to whether in the absence of the epithelial lobe the neurosecretory mechanism develops at all.

Methods. Tail bud embryos (Shumway Stage 17) of *Rana sylvatica* were used. The primordium at this stage is a small broadly-based projection of the deeper layer of the ectoderm ventral to the prosencephalon (Fig. 1). After removing the superficial layer of ectoderm a cut was made into the foregut cavity ventral to the primordium. The primordium was stripped from its contact with the brain from which it separates readily and cleanly except at the anterior dorsal aspect where the deep ectoderm fuses with the brain

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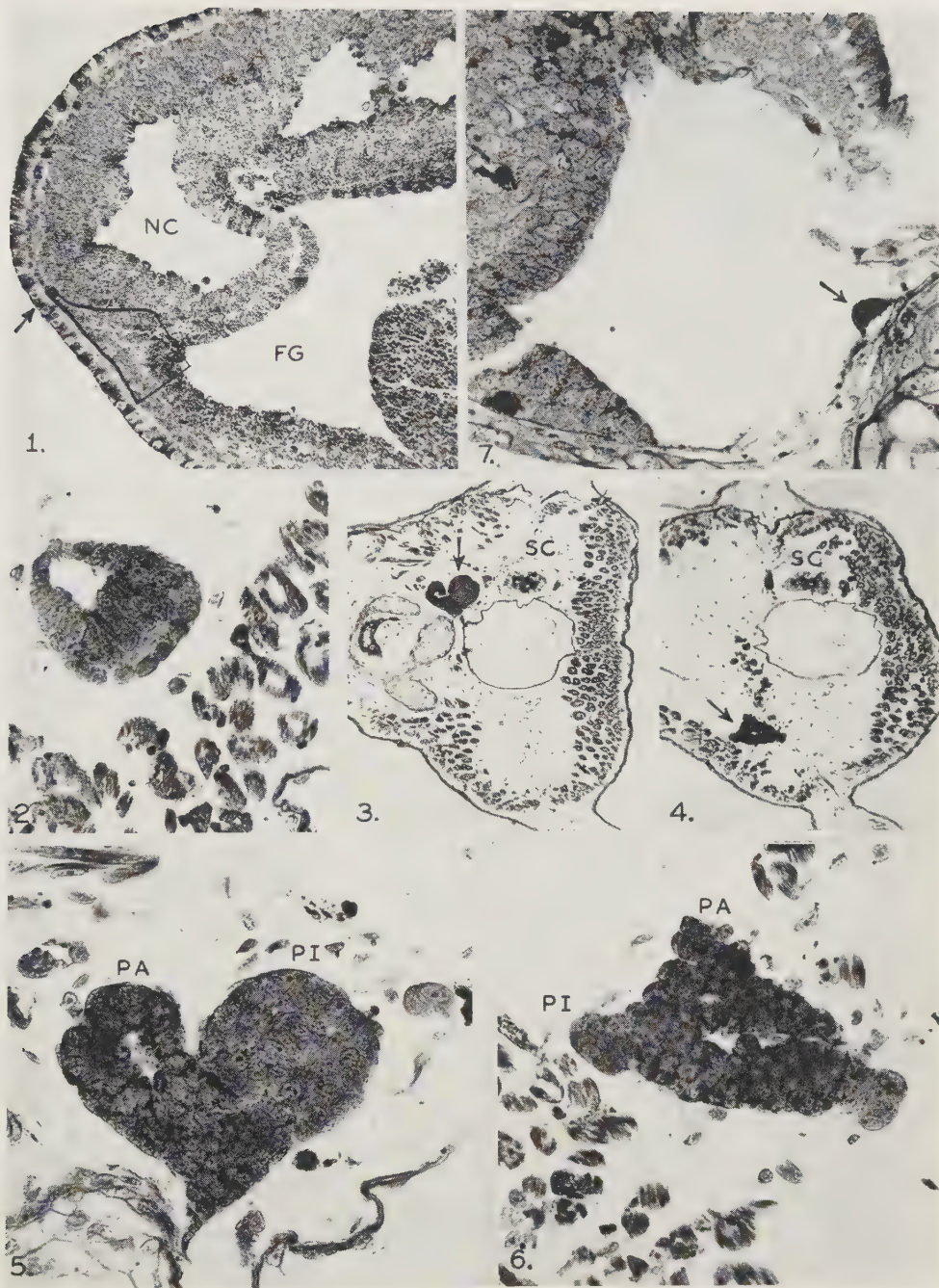


FIG. 1. Median sagittal section of Stage 17 embryo. Area taken in the graft is enclosed by the inked line. NC = neurocoel; FG = foregut. Arrow indicates region where deep ectoderm fuses with brain wall. Photograph 60 \times .

FIG. 2. Vesicle of neural tissue differentiated in one graft. Photograph 390 \times .

FIG. 3. Section through tail showing host spinal cord (SC) with graft (arrow) located nearby. Photograph 60 \times .

FIG. 4. Section through tail showing spinal cord (SC) and graft (arrow) located at considerable distance. Photograph 60 \times .

FIG. 5. Same graft as Fig. 3 at high power. PA = pars anterior; PI = pars intermedia. Photograph 390 \times .

FIG. 6. Same graft as Fig. 4 at high power. Photograph 390 \times .

FIG. 7. Sagittal section through infundibular region of donor animal showing neurosecretory mass at arrow. Photograph 390 \times .

TABLE I. Summary of Findings on Hosts to Grafts of Buccal Pituitary Primordium.

| Survivors to Stage 25 = 9 | Fixed at Stage 25 = 6 | | Fixed in late tadpole stage = 3 |
|---|-----------------------|-------------------|---------------------------------|
| Pigmentation at Stage 25— | Normal = 8; | Intermediate = 1; | Silver = 0 |
| Orthotopic pituitary— | Absent = 6; | Present = 2; | Undetermined = 1 |
| Graft pituitary | | | |
| 6 fixed at Stage 25 | P. ant. = 6; | P. intermed. = 6; | Degenerating tissue = 0 |
| 3 fixed tadpole stage | " = 1; | " = 1; | <i>Idem</i> = 2 |
| Graft nervous tissue | | | |
| 6 fixed Stage 25 | Absent = 5; | Present = 1 | |
| 3 fixed tadpole stage | " = 3? | " = 0? | |
| Graft position respecting host nervous system | | | |
| 6 fixed Stage 25 | Close = 1; | Distant = 5 | |
| 3 fixed tadpole stage | " = 0 | " = 3 | |

from which it had to be separated by cutting. (Arrow in Fig. 1). Unless the separation seemed clean-cut the specimen was discarded. The innermost cells of the primordium are fused with the endoderm. This endoderm was taken with the graft to avoid injury to the latter. For the same reason some of the lateral ectoderm was also taken. The primordium thus dissected was quickly inserted into a prepared opening in the tailbud of a previously hypophysectomized (*i.e.* epithelial primordium removed) host embryo of like size. The choice of the tailbud as the site of implantation was based on author's recent experience indicating that this region yields a high percentage of "takes." The dissection and transplantation were accomplished in a minute or less. The donor was then examined. If the brain floor appeared to have been damaged both donor and host were discarded. A further check on the accuracy of the dissection was afforded by keeping and studying the donors as well as hosts. Ten pairs of animals were prepared. Experimental animals were raised to the end of the embryonic period and beginning of feeding (Shumway Stage 25). The presence or absence of the pigmentary hormone of the pars intermedia can be definitely ascertained at this time by the state of dispersion of the melanophores and xantholeucophores. A silvery appearance of the animal, expanded xantholeucophores and contracted melanophores, indicates the absence of the pars intermedia in its entirety and of its hormone. By sacrificing all but 3 of the hosts at Stage 25 rather

than in late tadpole stages as in previous work it was hoped to avoid the possibility that some brain or gland differentiation had occurred but had been lost by degenerative changes before fixation. The hosts were killed in Helly's fixative. Heads and tails of hosts were sectioned and stained with Mallory's triple stain or with cresyl fast violet. The heads of donors were sectioned and stained with aldehyde fuchsin to demonstrate neurosecretory material.

Results. The pigmentary and histological findings on the host animals are summarized in Table I. It can be seen that all 9 surviving animals showed pigmentary evidence of the presence of pars intermedia secretion at normal or near normal levels. Since in 6 of these the animal's own pituitary was proved in sections to be absent, the pigmentary hormone must have been produced by the grafts. The histological findings showed that in all six animals fixed at Stage 25 both pars anterior and intermedia were differentiated in the graft. Five of these showed no nervous tissue present in the graft. In one a small vesicle of nervous nature (Fig. 2) was found in the graft area, but even here the vesicle was entirely separated from the pituitary tissue. One graft was found to lie close to a spinal root ganglion of the host (Fig. 3) but the 5 others were clearly and widely separated from cell bodies of the host nervous system (Fig. 4). If we exclude the animal showing the small neural vesicle, 4 of these six grafts must then be presumed to have developed without association with any nervous tissue

of graft or host. In 2 of the 3 animals fixed in late tadpole stages some degenerating tissue of uncertain nature was found. These animals do not therefore provide critical evidence on graft differentiation and need not be considered here in detail.

In sections the pars anterior of each of the Stage 25 grafts clearly shows characteristic tissue differentiation (Fig. 5, 6). The cells are small, arranged in compact cords and contain much embryonic pigment. No chromophilia is yet seen. In all respects, size, form and histological differentiation, the grafted pars anterior is entirely comparable to that of the normal gland at this stage of development. The pars intermedia is likewise readily identified in the grafts. The cells are large, with ample slightly basophilic cytoplasm (Fig. 5, 6). In 3 instances the size of the organ was very small though the tissue was cytologically well differentiated (Fig. 6). The microscopic structure of pars intermedia, except for size, was like that of the normal gland of the same stage. However, it suffered more of a loss in size on transplantation than did the pars anterior. This is the opposite of the condition found previously in long term grafts(11) where the pars intermedia was found to be hypertrophied and the pars anterior poorly developed. Two of the 3 animals kept to late tadpole stages showed excess body pigmentation. One of these subsequently became light in color and in section showed degenerating graft tissue. The other showed pars intermedia hypertrophy as previously reported.

The graft pituitaries generally lie in loose connective tissue usually in contact with host muscle fibers at some point. Other structures were found in the graft, such as cartilage, horny teeth, etc. as Eakin and others have reported. No consistent association of these tissues with the pituitary was noted but contact between pituitary and cartilage (Fig. 5) was most common.

The 9 surviving donor animals were all silver in color and upon sectioning were seen to lack entirely the epithelial pituitary. This indicates that the entire presumptive pituitary had been adequately removed in making the grafts.

In the normal animal at this stage the posterior tip of the infundibulum is formed by a very thin-walled vesicle continuous with the thin-walled roof of the infundibulum. A slight thickening in this vesicle in the region of its contact with the pars intermedia constitutes the early pars nervosa. With the aldehyde fuchsin technic a spot or a small diffuse area which stains intensely can be observed in this pars nervosa. This stained material presumably represents accumulated neurosecretory granules and serves to identify the pars nervosa. In the donor animals of this experiment the thin vesicular tip of the infundibulum is larger and more "blown-out" than normal. In 8 of the 9 specimens one to three spots of intensely staining material could be found at the tip of the infundibulum (Fig. 7). These are more concentrated than in the normal animal but clearly represent the accumulation of neurosecretory material as in the normal pars nervosa. The rest of the infundibular lobe of the brain is as large as in the normal animal but is abnormal in shape, and is lacking the clearly developed bilobed character found in the normal animal. This abnormality in shape has been known to characterize the infundibulum of the hypophysectomized animal since the original work of Smith(14).

Discussion. We conclude that in *Rana sylvatica* the epithelial primordium at the stage in which it first forms a recognizable thickening (Stage 17) is able to differentiate when transplanted to the tail to form both pars anterior and pars intermedia without further contact with the brain cells with which it normally is associated. It can so differentiate while lying in an area of loose connective tissue and muscle in which no host nerve cells are found. This conclusion is in agreement with most recent work(7,8) with respect to the pars anterior but not with respect to the pars intermedia. Since the other workers used different species it is possible that this divergence in results is due to a species difference in the time of embryonic determination in the primordium. In view of the use of Stage 18 animals of *Hyla regilla* by both Burch(4) and Eakin(7) which, as figured by Burch, have primordia even more advanced

than those used here, the differences in our results cannot be explained by my use of an earlier morphological stage. It is possible, therefore, that there is some difference between species in regard to differentiating capacities at the same stage, (stage 17-18).

In addition to species differences, however, there are differences in technic between the work reported here and that of others who have made transplants of primordia. This difference is most conspicuous regarding the degree to which the primordia are manipulated and regarding the site of implantation. I am inclined to view these differences as very important in determining the extent of development obtained although we have no way now of evaluating the relative importance of species as compared to technic differences. The point of view that I have adopted is that the graft should receive a minimum of handling and a maximum of protection by surrounding tissues. In the present series some tissue surrounding the primordium was taken on all sides except, of course, on the side in contact with brain. The small size of the surviving pars intermedia in 3 of the present grafts suggests that this tissue is especially vulnerable to injury in the course of the operation. The pars intermedia arises from the deepest cells of the invaginating primordium (15) and therefore would be most exposed to damage with resulting loss of cells. No attempt was made here to clean off neural cells from the transplant since such, if present, could not be distinguished from primordial cells. Manipulation was, therefore, as likely to remove cells of the primordium as it was to remove possible adherent nerve cells. The most satisfactory criterion of the presence or absence of nerve cells in the graft is the differentiation to be seen at the end of the embryonic period. The excellent differentiation attained by pituitary as well as other tissues in the tail bud location gives confidence in the use of this criterion. As indicated above, neural differentiation was absent in 5 of 6 animals fixed at Stage 25.

It should be noted that the presumptive pituitary cells originate in close association with the neural plate. The primordium from the time of its first appearance is in contact

with neural tissue. It has been isolated in the present experiment at a stage before it has sunken in deeply enough to reach the infundibular region of the embryonic brain (Fig. 1). It is possible, therefore, that inductive influences from the brain might have acted upon presumptive pituitary cells before Stage 17. If so, they must come from regions anterior to the infundibulum and act before the definitive pituitary primordium is formed in this species. The present experiment, therefore, does not exclude the possibility of an inductive influence of neural upon hypophyseal tissue. However, it does show that such influence, if present in this species, must come from the neural plate anterior to the infundibulum and act before the morphologically distinct primordium is formed.

The presence of typical masses of neurosecretory material at the tip of the infundibulum of the donor animals shows that even in the absence of the epithelial lobes the neurosecretory mechanism of the hypothalamus does differentiate. There is abnormality in the form of the infundibulum the significance of which remains to be elucidated. It should be noted that these animals were checked at operation and none showing injury to the brain were used. Furthermore, the grafts indicate that no nerve cells were taken with the primordium except in one case. The abnormality of the infundibulum cannot be ascribed to incidental injury to the brain, therefore, but is more likely to be related to the absence of the epithelial lobes.

Summary. The epithelial primordium of the pituitary in Stage 17 embryos of *Rana sylvatica* was removed without including or injuring adjacent brain and transplanted into the tail bud of hypophysectomized host embryos of the same stage. Donors and hosts were studied for pigmentary reaction and for histological differentiation of appropriate tissues. It was concluded that the epithelial primordium when it first appears as a morphologically distinct entity is capable of differentiating into both pars anterior and pars intermedia without further contact with brain or other nerve cells. It is likewise concluded that the neurosecretory mechanism of the hypothalamus differentiates normally in the ab-

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Interactions Between Mononuclear Phagocytes and *Brucella abortus* Strains of Different Virulence.* (23752)

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In prior studies on the multiplication of *Brucella abortus* in mononuclear phagocytes (monocytes) of guinea pigs, maintained *in vitro*, it was noted that after different periods of incubation some monocytes always tended to contain considerably more bacteria than others(1). It was suspected that such heterogeneity may have been due in part to a genetic heterogeneity among members of the bacterial population in regard to characteristics influencing ingestion by phagocytes and intracellular multiplication. It is well known that virulent smooth (S) and relatively avirulent rough (R) types are phagocytosed at different rates by polymorphs. In addition, it has been shown that nonsmooth *B. abortus* mutants fail to multiply within chick embryo fibroblasts capable of supporting multiplication of smooth type cells(2). Furthermore, when the culture used in the prior quantita-

tive studies(1) was examined 2 years later, appropriate tests(3) revealed that it contained approximately 20% R types in addition to S cells. Therefore, additional studies were initiated to determine the extent to which differences in antigenic and virulence characteristics of the bacteria may influence their interactions with monocytes from normal or immune guinea pigs.

Methods. Tissue culture technics followed essentially those detailed previously(1). Monocytes were harvested from guinea pigs 3-4 days following intraperitoneal injection of 2 ml sterile mineral oil. They were washed, counted and suspended in Hanks solution containing 30% homologous serum to yield a concentration of approximately 1×10^6 cells/ml. Bacteria harvested from 18-hour-old tryptose agar cultures were added to yield concentrations approximating either 2×10^8 /ml (*high* inoculum) or 2×10^7 /ml (*low* inoculum). 1.5 ml of such suspensions were placed into rubber-stoppered Porter flasks containing flying cover slips, and incubated at 37°C. Two hours later the liquid was replaced by serum-Hanks' containing 10 µg of streptomycin per ml. It was established that this concentration of antibiotic will kill all except <0.001% of the extra-cellular bacteria

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TABLE I. Data Obtained from One Maintenance Culture of Normal Guinea Pig Monocytes Exposed to a Mixed S + R Population of *B. abortus*, 6232.

| Bacterial counts | % S |
|--|-----|
| Initial (F*): 2×10^8 | 55 |
| From supernate of 72-hr-old culture (L*) approx. 2000 | 64 |
| From supernate of harvested washed mono's (L), approx. 300 | 79 |
| Within mono's, final (F): 5.5×10^5 | 90 |
| <i>Microscopic observations</i> | |
| Infected mono's: 98% | |
| Mono's with >10 bacteria: 65% | |
| " " >25 " 41% | |

* F = per flask; L = per loopful.

within 2-6 hours but, apparently, will not interfere with their intracellular multiplication. After a given period of incubation (usually 24, 48, or 72 hours after introduction of the streptomycin-serum-Hanks' medium), cover slips were removed from the Porter flask cultures and stained; the remaining monocytes were harvested and washed as previously reported(1). Samples from the supernatant (*Su*) of washed, centrifuged monocytes were

plated on 2-1 agar(4) for determination of colonial morphology of the bacterial progeny, and viable counts were made by plating appropriate dilutions on tryptose agar. The final bacterial count resulted from suspending and diluting the washed, sedimented monocytes in *plain* Hanks' which apparently sufficed to release the intracellular bacteria from the leukocytes. Table I and Fig. 1 indicate the kind of data collected for each of the 100 monocyte cultures on which the present report is based. The bacterial strains employed in this study included either S or R types isolated from an air-growing culture of *B. abortus*, SA, and from a CO₂-requiring culture of *B. abortus*, 6232. The SA culture was originally isolated from a human case in Puerto Rico, and *B. abortus* 6232 originated from an infected cow. In addition, a smooth *B. abortus*, strain 19, culture was used in a few experiments.

TABLE II. Final Bacterial Count ($\times 10^3$) per Flask after 48 Hours of Incubation.

| Bacterial inoculum | Exp. #5 | Exp. #7 |
|-----------------------|---------|----------|
| SA-S | 352.1 | 90.0 |
| -R | 7.3 | 3.5 |
| 6232-S | 4.8 | not done |
| -R | .6 | " |
| -S in CO ₂ | 20.8 | " |
| -R " | 9.0 | " |

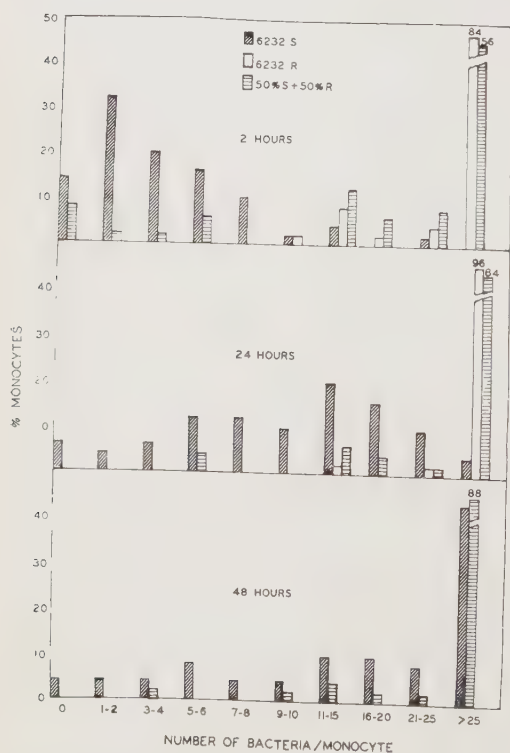


FIG. 1. Distribution of number of *B. abortus* per monocyte after various periods of incubation.

Results. Differences between S and R strains. A large series of bacterium-monocyte cultures, initiated either with SA-S, SA-R, 6232-S, 6232-R, or mixtures of corresponding S + R bacteria, indicated that virulent S cells multiplied in monocytes from normal guinea pigs, whereas the relatively avirulent R type cells either failed to do so or multiplied only to a limited extent. Such differences in the capacity for intracellular growth were detectable by comparison of the final viable counts of bacteria (Table II), by the pattern of distribution of number of bacteria per monocyte after various periods of incubation (Fig. 1), by comparing (Table III) the relative ratio of bacteria harvested from disintegrated monocytes (final count) to number of extracellular bacteria recoverable from the supernatant (*Su*, see methods), and by comparison of the ratio of the final count

TABLE III. Ratio of Number of Bacteria Harvested from Washed Monocytes to Number Recoverable from Supernatant.
(Age of cultures: 72 hr.)

| Bacterial inoculum | | Ratio |
|--------------------|--------|-------|
| SA-S | "high" | 51.4 |
| -R | " | 6.1 |
| 6232-S | " | 5.9 |
| -R | " | 2.3 |
| Strain 19-S | " | .8 |
| SA-S | "low" | 10.9 |
| -R | " | 3.8 |
| 6232-S | " | 5.4 |
| -R | " | 2.7 |

from 48-hour-old cultures with the final count from corresponding 2-hour-old cultures (Table IV). In addition, the increase in % S in bacterial populations harvested from cultures originally inoculated with a mixture of S + R (Table I) reflected the differential multiplication of virulent and relatively avirulent types.

Observations on the distribution of number of bacteria per monocyte showed significant differences between S- and R-exposed cultures, particularly during the early stages of bacterium-monocyte interactions. As illustrated in the "2-hour" example of Fig. 1, the majority of monocytes exposed to an excess of S bacteria contained 1-6 bacteria shortly after initiation of the Porter flask cultures; in contrast, monocytes exposed to R bacteria were virtually loaded with bacteria at this stage. Such differences can be attributed to a number of causes, including the tendency of R type bacteria to clump and to be ingested with greater ease than S bacteria. In addition, this difference may be indicative of some type of change occurring in monocytes that have ingested one S bacterium, which may interfere with the continued ingestion of additional S bacteria during the first 2 hours. However, regardless of the actual causes for the early differences in number of S *vs.* R bacteria per monocyte, the existence of such pronounced differences seems to permit their utilization for the simple and rapid detection of the extent of homogeneity or heterogeneity existing in the bacterial population that was employed as inoculum (see

2-hour data in Fig. 1, including the bimodal distribution in the case of a mixed S + R inoculum). The described differences tend to disappear with increasing time of incubation (Fig. 1) due to a gradual shift to higher numbers of bacteria per monocyte in cultures with S bacteria; this shift undoubtedly is a reflection of the intracellular multiplication occurring in such S cultures.

One additional phenomenon is reflected in Fig. 1 by the absence of any 48-hour counts for monocyte cultures initially exposed to R bacteria. The lack of such determinations is due to the almost total absence of nondisrupted monocytes, which reflects the rapid rate of destruction of monocytes following ingestion of large numbers of R bacteria. Many of the S-containing monocytes also eventually disintegrated, but this did not occur until later periods when large numbers of bacteria per monocyte had been attained due to intracellular multiplication.

Differences between S strains. In all experiments, strain SA-S displayed the most rapid rate of intracellular multiplication during the first 48 hours. Bacteria of strain 6232 S (CO₂-requiring) multiplied at a considerably slower rate in rubber-stoppered cultures, and this tardy rate was not brought up to that of SA-S even when cotton-plugged cultures were incubated in CO₂ (Table II). However, after 72 hours of incubation (see "Normal" data in Table V), the CO₂-requiring bacteria appeared to catch up with the non-CO₂ requirers as far as final counts obtained from cultures showing similar survival of monocytes are concerned. Microscopic observations of monocytes from representative cultures have revealed no significant differences in rate and extent of ingestion of 6232-S or SA-S bacteria. Therefore, such observations would indicate that the observed strain dif-

TABLE IV. Ratio of Final Bacterial Count from 48-Hour-Old Cultures to Final Count from 2-Hour-Old Cultures.

| Bacterial inoculum | | Ratio |
|--------------------|--------|-------|
| SA-S | "high" | 4.6 |
| -R | " | .05 |
| -S | "low" | 8.3 |
| -R | " | .27 |

TABLE V. Final Bacterial Count ($\times 10^8$) per Flask in 72-Hour-Old Cultures Initiated with Immune or Normal Monocytes.

| Bacterial inoculum | Monocytes and serum | Count |
|--------------------|---------------------|-------|
| SA-S | Normal | 317.5 |
| " | Immune | 13.0 |
| 6232-S | Normal | 375.0 |
| " | Immune | 1.5 |
| SA-R | Normal | 15.0 |
| " | Immune | .8 |
| 6232-R | Normal | 30.0 |
| " | Immune | 1.3 |

ferences are due primarily to differences in the duration of the lag period prior to initiation of intracellular multiplication. It remains to be determined to what extent these differences may be associated with strain differences in virulence. S (perhaps more properly labelled I_1) bacteria from the immunogenic, relatively avirulent *B. abortus*, strain 19, displayed an exceptional behavior. They failed to multiply intracellularly and appeared to disintegrate rapidly in normal guinea pig monocytes. This is in contrast to the microscopically visible retention of morphological integrity of other S and R bacteria studied. However, there is a resemblance between the morphological and physiological interactions of strain 19 bacteria with *normal* monocytes, and the interactions observable when bacteria from virulent strains are ingested by monocytes from *immune* guinea pigs.

Interactions with immune monocytes. A limited number of cultures containing immune serum were initiated with monocytes from immune (previously infected) guinea pigs and S or R bacteria. In all instances ingestion of bacteria was fast, and was followed by very rapid bacterial and cellular destruction. Little or no intracellular multiplication of S bacteria occurred (Table V). There was some indication that, in contrast to the effects noted with normal monocytes, S bacteria may destroy immune monocytes more rapidly than R bacteria.

Miscellaneous observations. With an increase in the size of the bacterial inoculum, increases in the extent of ingestion, rate of monocyte destruction and final counts after a given period of time were observed. In ad-

dition, it was noted that quite frequently a large proportion of bacteria harvested from monocyte cultures gave rise to tiny colonies; this phenomenon probably is indicative of some temporary modification (injury) suffered by the clonal parent since subcultures from these tiny colonies yielded colonies of normal size.

Discussion. The foregoing observations indicate that S and R types of *B. abortus* differ not only in their capacity for intramonocytic multiplication, as they do in the case of chick fibroblasts(2), but that they also differ in regard to the rate with which they are ingested and with which they subsequently destroy monocytes. The low initial extent of multiple ingestion of S bacteria by individual monocytes may be due either to a specific interference with continuous ingestion or merely to an injury to the phagocyte caused by prior contact with an S type cell. In contrast, the less virulent R type bacterium may be less injurious and thus leave the phagocyte with the continued capacity for ingestion. "Superinfection" experiments with labelled S and R strains will be required in order to elucidate this point. The more rapid destruction of normal monocytes by R than by S bacteria may be a reflection of the need for attainment of a minimum number of bacteria per normal monocyte prior to initiation of monocyte destruction. The required intracellular number of bacteria would be attained rapidly by continued early ingestion in the case of R types, but more slowly by intracellular multiplication in the case of S types. An even more rapid destruction of monocytes that was observed to occur following exposure to S + R mixtures, possibly due to the combined effects of intracellular multiplication (S) and initial multiple ingestion (R).

The observed alterations of bacterium-monocyte interactions *in vitro* due to differences in bacterial population heterogeneity, strains, and inoculum sizes, stress the need for a certain degree of standardization of procedures and materials as a prerequisite for comparing results obtained in different laboratories.

It is noteworthy that even following the use of relatively homogeneous bacterial in-

ocula, there remained a certain amount of bimodality in the distribution of number of bacteria per monocyte in cultures older than 24 hours. This would suggest that in the present and past(1) studies some unrecognized heterogeneity persisted either in the smooth bacterial populations (in regard to properties influencing interactions with mammalian cells) or in the phagocyte population. Preliminary experiments have indicated that differences in the age of individual monocytes at time of initiation of the cultures may account at least in part for such heterogeneity.

Finally, the observations on monocyte destruction *in vitro* suggest certain future studies in regard to the extent to which similar phenomena *in vivo* might modify the course of disease caused by facultative intracellular bacteria such as *Brucella*. Since it is likely that for these organisms residence and multiplication within phagocytes represents an advantage for the pathogen (*e.g.*, protection from bactericidal blood factors and certain antibiotics), induced monocyte destruction by avirulent R type cells, possibly even by appropriate inert particles or other methods, may contribute to the amelioration of the disease. It is possible that such an approach already may have been employed unwittingly in the case of combined tuberculin and antibiotic treatment(5).

Summary. Studies on the *in vitro* interactions between S and R strains of *Brucella*

abortus and monocytes from normal or immune guinea pigs have indicated 1) intracellular multiplication of S bacteria and a lack of, or insignificant extent of, multiplication of R bacteria in normal monocytes, 2) differences in the rate of ingestion of S and R bacteria, 3) differences in the rate of destruction of monocytes by S and R types, 4) differences in the rate of intracellular multiplication among S strains, 5) a significant modification of these events in immune monocytes, and 6) an apparently unique behavior of immunogenic, relatively avirulent strain 19-S bacteria in normal monocytes. The relation of these observations to some problems of host-parasite interactions is discussed. It also has been shown how these observations may provide the basis for a new technic of rapid assessment of the homogeneity or heterogeneity of bacterial populations in regard to properties associated with virulence.

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Leukemia. VIII. Leukemogenic Effect of Brain Filtrates After Serial Passage Through Mice.* (23753)

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Because of the purported ability of cell-free brain extracts to induce leukemia in mice (1-4), experiments were undertaken to test further the validity of the claim that the filtrates were cell-free. The experiments were so devised that they would give evidence also

of the presence of a self-perpetuating agent, and would allow for the comparison of cell-free brain filtrates with tumor homogenates as leukemogenic agents.

The demonstration by Furth(5) that a single cell injected intravenously may produce a lymphoblastoma in mice and the reports of Stasney, Cantarow and Paschkis(6,7)

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that even cell particles could produce tumors made it mandatory to demonstrate that cellular contamination could not be held responsible for the results reported. This was made even more important since the interval between inoculation of the animals and development of the lymphoblastoma is of the same order of magnitude whether cell-free filtrates or whole-cell suspensions are used as inocula(3).

Materials and methods. The AKR mice (high leukemic strain) used in the experiments were obtained from The Jackson Memorial Laboratory. The C3Heb mice (very low leukemic strain) were bred from a pedigreed breeding stock originally obtained from The Jackson Memorial Laboratory. The Swiss mice (low leukemic strain) were obtained from Harlan Small Animal Industry, Cumberland, Ind. Filtrates were prepared by a slight modification of the method previously described(1). The brains were ground with sand in a mortar in an ice water bath. The sand was allowed to settle. The supernatant was then centrifuged at 2,500 rpm for 15 minutes, decanted and passed through a Seitz sterilizing pad whose integrity was tested with a saline suspension of *E. coli*. 250 mg of mouse brain or human brain are used per hundred cubic centimeters of saline solution in preparing the cell-free filtrate, and of this 0.5 cc is used for the intraperitoneal and 0.1 for the intracerebral injection. The experiments were so devised that the original material, whether cell-free filtrate of brain or tumor homogenate, was introduced into a heterologous strain with a low incidence of spontaneous leukemia. In the case of the cell-free filtrates of brain, the material was preferentially introduced intraperitoneally in order to invalidate the possible objection that previously inoculated material was simply reharvested and passed in subsequent passages. Nonetheless some intracerebral inoculations were made for comparative purposes. The experiments with tumor homogenates were uniform in that the material was injected intraperitoneally. In the case of the tumor homogenates, parallel experiments were performed with the use of the cell-free extracts

of brains from the same mice that served for the tumor source. The heterologous strain mouse was killed 3 days after it had been inoculated and its brain was used for the preparation of a new filtrate, which in turn was inoculated intraperitoneally into heterologous mouse No. 2. Thereafter the process was repeated until the cell-free extract of the brain of mouse No. 5 was prepared. This filtrate was injected intracerebrally into groups of 20 or 30 mice of the original (homologous) strain. These mice were from 9 to 12 weeks old and served as the test animals. Human brain material was identically treated except that Swiss mice served as the passage, and AKR as the test animals. *Heat inactivated filtrates* and filtrates prepared from nonleukemic mouse and human brain were used for controls and were similarly passed. Two additional control experiments were carried out: In these, tumor cell suspensions of Swiss leukemia were prepared by grinding leukemic lymph nodes and tumor tissue in a ground glass tissue mill with 0.85% solution of sodium chloride; 0.5 cc of a 10^6 cells per cc suspension were injected intraperitoneally into C3Heb mice at the start of the serial passage. At the same time, cell-free filtrates prepared from the brains of the same mice that served as a source of the tumor were passed

TABLE I. Results of Serial Passage of Brain Filtrates.

| Original source | Route of passage | Passed through | No. mice with leuk./total inj. |
|--------------------|------------------|----------------|--------------------------------|
| C.F.F. of brain | | | |
| <i>Swiss mouse</i> | | | |
| Leukemic | I.C. I.P. | C3Heb | 8/20 (Swiss) 16/20 |
| Nonleukemic | I.C. I.P. | | 0/30 " |
| <i>AKR mouse</i> | | | |
| Leukemic | I.C. I.P. | Swiss | 19/20 (AKR) 17/20 |
| Nonleukemic | I.C. | | 0/20 |
| <i>Human</i> | | | |
| Leukemic | I.C. I.P. | " | 19/20 " |
| Nonleukemic | I.C. I.P. | | 0/20 " |

C.F.F. = Cell-free filtrate.

I.C. = Intracerebral; I.P. = Intraperitoneal.

TABLE II. Comparison of Leukemogenic Effect of Brain Filtrates and Tumor Cell Suspensions after Serial Passage.

| Original source | Route of passage | Passed through | No. of Swiss mice with leuk./total inj. |
|-----------------------------------|------------------|----------------|---|
| <i>Leukemic Swiss mouse No. 1</i> | | | |
| C.F.F. brain | I.P. | C3Heb | 9/20 |
| Tumor-cell susp. | " | | 0/20 |
| C.F.F. brain, heat inactivated | " | | 0/30 |
| <i>Leukemic Swiss mouse No. 2</i> | | | |
| C.F.F. brain | " | " | 8/20 |
| Tumor-cell susp. | " | | 1/20 |
| C.F.F. brain, heat inactivated | I.C. | | 0/30 |

C.F.F. = Cell-free filtrate.

I.C. = Intracere; I.P. = Intraper.

through C3Heb mice. After 5 passages, filtrates from both lines were injected into Swiss mice intracerebrally.

Results. The results of the experiments with the brain filtrates from the various sources are shown in Table I. The results of the experiments in which a comparison was made between cell-free filtrates of brain and tumor-cell suspension from the same animal are shown in Table II.

Discussion. The experiments described demonstrate that contamination by cells or cell fragments is *not* the explanation for the results previously reported. If one assumes 100% recovery of the material which is injected intraperitoneally in each passage, the effect of 5 passages is to dilute the original material to approximately 10^{-13} . The activity of the original filtrate is lost when diluted to 10^{-5} . It is, therefore, necessary to assume that the activity has been replicated.

The experiments in which cell-free brain filtrates and tumor homogenates served as parallel starting substances further underline two points previously emphasized: one, that it

is not a cellular or cell fragment contamination that is responsible for the results, since in the case of the tumor homogenate infinitely more cells serve as starting points than could ever be assumed as accidental contaminants, and second, that the active agent is present in the brain in a concentration far greater or a form far more active than that found in tumor tissue or leukemic lymph nodes.

Summary. 1. Cell-free filtrates of leukemic AKR and Swiss mouse and human brains were prepared. 2. The filtrates were serially passed through Swiss mice in the case of AKR mouse and human brains and through C3Heb mice in the case of Swiss mice. 3. After 5 passages leukemogenic activity could be demonstrated by reintroduction of the material into the starting strain in case of the mice, and into AKR mice in case of human brains. 4. No activity or little activity could be demonstrated when leukemic tissues served as sources of the original material. 5. The experiments demonstrate that the activity of the material is not dependent on cells. 6. The dilution resulting from the passages described is such as to compel the assumption that the activity has been replicated.

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Dependence of Protoveratrine-induced Hypotension on the Hypothalamus.* (23754)

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Through numerous studies (see Martini and Calliauw(1) for the literature) it has been established that certain veratrum derivatives including protoveratrine produce a hypotension through the excitation of the baroreceptors of the sino-aortic area. In a series of papers from this laboratory(2-9) the relation of baroreceptor reflexes to the hypothalamus was investigated by determining the action of hypotensive drugs (acetylcholine, mecholyl, histamine) and hypertensive agents (noradrenaline and adrenaline) before and after the excitability of the hypothalamus had been altered by various procedures. The resulting changes particularly in the cardiovascular action of these substances indicated that: 1. the noradrenaline-induced pulse slowing reflex depends, other conditions being equal, on excitability of the anterior hypothalamus; 2. intensity of sympathetic reflexes elicited by peripheral action of acetylcholine and related drugs depends on excitability of sympathetic division of the hypothalamus involving lateral and posterior hypothalamic nuclei.

Whereas noradrenaline and acetylcholine and related drugs cause a change in intensity of the baroreceptor discharges through variations in blood pressure and consequently in pressure of the sino-aortic area, protoveratrine alters these discharges directly. Injection of veratrum derivatives into the adventitial layer of the bifurcation of the carotid artery(10) or local application of this drug(11) elicits a fall of the blood pressure provided the baroreceptors are intact. The experiments here described were designed to determine whether the protoveratrine-induced baroreceptor reflexes depend likewise on the state of excitability of the hypothalamus.

Methods. The experiments were performed on 21 cats prepared under local anes-

thesia supplemented by thiopental i.v. Hess electrodes were inserted permitting either injection of pentobarbital or coagulation of a part of the hypothalamus through high frequency currents. The blood pressure was recorded from the femoral artery. EEG records were taken at the same time. Protoveratrine, kindly supplied by E. R. Squibb and Sons, was injected i.v. 1-2 hours after the operation while the cat was under artificial respiration and intocostirin. For further details see the above cited papers. Coagulation and histology as in our earlier work(12).

Results. Table I shows that the average fall of blood pressure following injection of Protoveratrine was 37 mm Hg in the control group, 4.5 mm after injection of 0.02 cc pentobarbital intrahypothalamically, and 8 mm after bilateral coagulation in the hypothalamus. The 2 experimental groups (10 cases) were combined and compared with the control group (11 cases). The difference is statistically highly significant ($\chi^2 = 9.39$; $p < 0.01$). Testing the difference between the means yields $t = 4.26$; $p < 0.001$.

Fig. 1 shows the sites of the injections projected on the median sagittal plane although

TABLE I. Fall of Blood Pressure (in mm Hg) after Intravenous Injection of Protoveratrine (.0005 mg/kg).

| A | After inj. of Nembutal* in lateral hypothalamus | After bilateral coagulation in lateral hypothalamus | After inj. of Dial intraper. |
|----------|---|---|------------------------------|
| Controls | | | |
| 56 | 0 | 8 | 6 |
| 27 | 25 | 10 | -8 |
| 22 | 0 | 6 | 8 |
| 86 | -3 | 8 | 5 |
| 54 | 5 | | |
| 10 | 0 | | |
| 35 | | | |
| 10 | | | |
| 30 | | | |
| 27 | | | |
| 50 | | | |
| † 37 | 4.5 | 8.0 | 2.8 |

* These studies were aided by grant from W. Louis and Maud Hill Family Fn.

* .02 cc 6%.

† Avg.

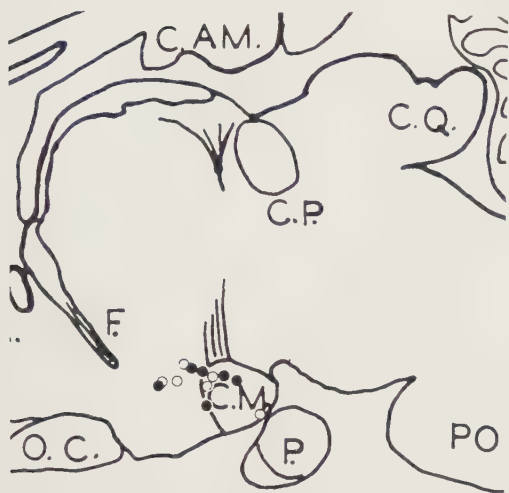


FIG. 1. Sagittal section through brain of cat. Black dots indicate site of inj. at left side, circles on right side. Site of inj. was 1-3 mm lateral to the median plane. O.C., optic chiasma; C.M., mammillary body; P., pituitary; F., fornix; PO., pons.

they were 1-3 mm lateral to it. They are closely related to the mammillary bodies and affect chiefly the lateral hypothalamic nuclei. The sites are similar to those which caused a fall of the blood pressure and pulse rate on intrahypothalamic injection of barbiturates or procaine(5).

The chief site of the lesions was in the same area. The mammillary bodies and the lateral and posterior hypothalamic nuclei were mainly involved. In one instance the lesion extended to a part of the red nucleus and, in another case, to one side of the supraoptic nucleus.

On the basis of earlier work from this laboratory it may be said that these injections and lesions interfere primarily with sympathetic excitability and tonic sympathetic impulses at the hypothalamic level. Since Protoveratrine fails to evoke a hypotension in sympathectomized animals(10) the action of the drug appears to be due to an inhibition of the central sympathetic tone. Our experiments seem to indicate that an important site of this inhibitory action is the sympathetic division of the hypothalamus.

Table II shows the reversibility of the action of the intrahypothalamically injected barbiturates. Whereas Protoveratrine is practically ineffective following this injection, it causes a distinct hypotension one or 2 hours later. At this time the excitability of the hypothalamus to direct stimulation was completely or almost completely restored to the control level.

The experiment on cat 5094 illustrates the fact that although 0.0005 mg Protoveratrine fails to induce a hypotension in a cat with an appropriate hypothalamic lesion, a sufficiently increased dosage (0.003 mg/kg) occasionally evokes a rise in the blood pressure. The central excitatory action of Protoveratrine on the medulla oblongata and spinal cord(10,13,14) which appears in the dog after sino-aortic denervation is retained in the cat in which, due to a lesion in the hypothalamus, the sino-aortic reflexes had been weakened.

Comment. To demonstrate the importance

TABLE II. Effect of Increasing Doses of Protoveratrine on Blood Pressure in Cats with Reduced Hypothalamic Excitability.

| Cat # | Protoveratrine (mg/kg intrav.) | Blood pressure | | Difference (mm Hg) | Remarks |
|-------|-----------------------------------|----------------|---------------|-----------------------|------------------------|
| | | Before inj. | After inj. | | |
| 5094 | .0005 | 135 | 108 | 27 | Control |
| | .0005 | 112 | 106 | 6 | Bilateral coagulation* |
| | .0015 | 108 | 100 | 8 | <i>Idem</i> |
| | .003 | 93 | 100 | -7 | " |
| 5120 | .0005 | 125 | 120 | 5 | Nembutal bilaterally* |
| | .001 | 135 | 125 | 10 | <i>Idem</i> † |
| | .001 | 120 | 85 | 35 | " (1 hr later) |
| | .0005 | 120 | 105 | 15 | " (" ") |
| 5121 | .0005 | 122 | 122 | 0 | Nembutal bilaterally* |
| | .001 | 120 | 120 | 0 | <i>Idem</i> † |
| | .001 | 136 | 106 | 30 | " (2 hr later) |

* In posterior hypothalamus.

† The first 2 tests on cat 5120 and 5121 were performed within 10 min. after intrahypothalamic inj. of Nembutal.

of the hypothalamus for sino-aortic baroreceptor reflexes it is necessary to use animals in which the effect of the barbiturates administered during the operation had worn off. This was indicated by the absence of slow potentials in the EEG of our animals. If, however, the experiments are performed in deep barbiturate anesthesia, the hypotensive action of Protoveratrine is absent as in the tests done under Dial-urethane anesthesia (Table I). Under these conditions the excitability of the posterior hypothalamus is greatly reduced(15) as in experiments involving lesions or intrahypothalamic injections of thiopental in this area.

The conclusion derived from our earlier studies cited in the introduction that baroreceptor reflexes act on the hypothalamus is confirmed by the experiments on the action of Protoveratrine described in this paper. Previously published data and our own show that small doses of Protoveratrine (0.0005 mg/kg i.v.) elicit a hypotensive effect in the "light" cat but not in barbiturate anesthesia. Moreover, lesions in the sympathetic division of the hypothalamus or reduction of its excitability by the injection of minute quantities of barbiturates likewise reduce or eliminate the hypotensive action of Protoveratrine. Bearing in mind the action of Protoveratrine on the baroreceptors, it may be said that the hypothalamus is more sensitive to baroreceptor reflexes than the medulla oblongata.

Summary. In view of the fact that baroreceptor reflexes depend on excitability of the hypothalamus and that Protoveratrine elicits these reflexes, the action of Protoveratrine in

relation to hypothalamic excitability was investigated in the cat. It was found: 1. That injection of barbiturates into the lateral and posterior hypothalamus practically abolishes the Protoveratrine-induced hypotension. This effect is reversible. 2. That lesions confined chiefly to the lateral hypothalamus exert a similar but irreversible effect. Such lesions do not interfere with the hypertensive action of larger doses of Protoveratrine. 3. That barbiturates administered intraperitoneally in anesthetic concentrations eliminate the hypotensive action of Protoveratrine.

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Tissue Lipids of *Dystrophia muscularis*, a Mouse with Inherited Muscular Dystrophy.* (23755)

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A rise in cholesterol and total lipid content of skeletal muscle is a prominent feature of nutritional muscular dystrophy induced by the feeding of a Vit. E-deficient ration to guinea pigs, rabbits, rats and calves(1-5). In the rabbit this is accompanied by a hypercholesterolemia(1), and in both the rat and the rabbit increases in the cholesterol content of the brain have been reported(2,4). Increases in the cholesterol level of the muscle have been found in Vit. E-deficient chicks(6) and in patients afflicted with muscular dystrophy(7). Hitherto it was not possible to obtain laboratory animals with dystrophy without a concomitant deficiency in Vit. E. Recently *Dystrophia muscularis*, a mutation in strain 129 mice, with inherited muscular dystrophy, became available(8). Since investigations of the nature of the disturbance in cholesterol metabolism of dystrophic muscle might reveal the underlying metabolic disorder in this disease, in this communication are reported the cholesterol and total lipid content of tissues of *Dystrophia muscularis*.

Procedure. Dystrophic mice and their non-dystrophic littermates were sacrificed under nembutal anesthesia. Blood samples were obtained by heart puncture. Pooled blood samples were centrifuged, and the lipids were extracted from the plasma with ethanol:ace-

tone (1:1). Skeletal muscle, liver and brain were analyzed individually; and kidneys, heart and lungs from animals of the same sex were pooled for analysis. Skeletal muscle, heart and lungs were finely ground with acid-washed sand and anhydrous sodium sulfate; and liver, brain and kidneys were ground with anhydrous sodium sulfate alone. The resulting dry powder was then extracted with boiling ethanol:petroleum ether (skellysolve B) (3:20), the supernatant decanted, and the residue extracted twice more with fresh portions of boiling petroleum ether; the 3 extracts were then combined. All extracts were analyzed for cholesterol by a modification of the Schonheimer-Sperry procedure reported by Niefert and Deuel(9). Total lipids were determined gravimetrically on the petroleum ether extracts of the original tissues.

Results. In Table I are presented the cholesterol and total lipid content of skeletal muscle of dystrophic and non-dystrophic mice. In the dystrophic animals, there is an elevation of muscle cholesterol over that of the normal control; this is in agreement with earlier findings, that a rise in muscle cholesterol obtained in muscular dystrophy(1-5,7). However, in only the dystrophic female mice is there also a significant increase in muscle total lipid.

The plasma cholesterol levels (Table II)

TABLE I. Free Cholesterol and Total Lipid Content of Skeletal Muscle of Dystrophic and Non-dystrophic Mice.

| Group | | No. of determinations | Free cholesterol, mg/g wet wt | p* | Total lipid, g/100 g wet wt | p* |
|-------|----------------|-----------------------|-------------------------------|------|-----------------------------|---------------|
| ♂ | Dystrophic | 7 | 1.46 ± .14 † | <.01 | 10.7 ± 5.4 † | .3 > p > .2 |
| ♂ | Non-dystrophic | 7 | .925 ± .028 | | 7.70 ± .68 | |
| ♀ | Dystrophic | 7 | 1.59 ± .06 | " | 11.2 ± 1.2 | .02 > p > .01 |
| ♀ | Non-dystrophic | 6 | .954 ± .067 | | 6.50 ± .96 | |

* Probability that difference between mean values for dystrophic and non-dystrophic is due to chance.

† ± stand. error.

* This investigation supported by grant from Muscular Dystrophy Assn.

† Facilities of Allan Hancock Fm. are gratefully acknowledged.

TABLE II. Cholesterol Content of Plasma of Dystrophic and Non-dystrophic Mice.

| Group | No. of determinations | Cholesterol | |
|----------------|-----------------------|-----------------|----------------|
| | | Free, mg % | Total, mg % |
| Dystrophic | 7 | 46.8 \pm 6.5* | 126 \pm 20 * |
| Non-dystrophic | 7 | 43.6 \pm 3.4 | 127 \pm 9.5 |

* \pm stand. error.

show no changes in the dystrophic animal as compared with the non-dystrophic mouse. These findings are similar to those obtained in children with muscular dystrophy where the plasma cholesterol is, in fact, slightly lower than normal(10), but are in contrast with results reported from dystrophic rabbits where a hypercholesterolemia obtains(1).

In Table III are the cholesterol and total lipid levels of liver, brain, kidney, lung and heart. In neither male nor female dystrophic mice is there any alteration in the cholesterol and total lipid content of brain and liver. In dystrophic males there is a slight increase in the cholesterol concentration of the kidneys and lungs which is not observed in dystrophic females, whereas in dystrophic females, there is an elevated cholesterol content of the heart. As these changes in the cholesterol content of

heart, kidney and lung are of lesser magnitude than those in muscle and are not noted in both sexes, they are probably of doubtful importance. Total lipid values in these tissues are unaffected.

That the only consistent increase in tissue cholesterol in muscular dystrophy is in skeletal muscle indicates that the aberration of cholesterol metabolism in *Dystrophia muscularis* resides in the muscle itself or in some mechanism responsible for lipid transport to or from the muscle. The possibility also exists that the elevated muscle cholesterol arises as a result of degenerated muscle fibers. Experiments are now in progress in an attempt to elucidate the nature of this disturbance in cholesterol metabolism.

Summary. 1) Tissues of mice with hereditary muscular dystrophy and their normal litter-mates have been analyzed for cholesterol and total lipid content. 2) There is a consistent and highly significant increase in cholesterol concentration in the muscle of dystrophic mice of both sexes over that found in the non-dystrophic control animals.

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TABLE III. Cholesterol and Total Lipid Content of Liver, Brain, Kidney, Lung and Heart of Dystrophic and Non-dystrophic Mice.

| Tissue | Group | No. of determinations | Cholesterol | | Total lipid, g/100 mg wet wt |
|--------|------------------|-----------------------|-------------------|--------------------|------------------------------|
| | | | Free, mg/g wet wt | Total, mg/g wet wt | |
| Liver | ♂ Dystrophic | 7 | 2.75 \pm .15* | 3.46 \pm .75* | 5.20 \pm .15* |
| | ♂ Non-dystrophic | 7 | 2.50 \pm .06 | 3.35 \pm .10 | 5.10 \pm .15 |
| | ♀ Dystrophic | 7 | 2.86 \pm .18 | 4.06 \pm .40 | 5.50 \pm .46 |
| | ♀ Non-dystrophic | 6 | 2.68 \pm .28 | 3.77 \pm .17 | 5.50 \pm .46 |
| Brain | ♂ Dystrophic | 7 | 12.5 \pm .58 | 12.5 \pm .55 | 8.04 \pm .18 |
| | ♂ Non-dystrophic | 7 | 13.6 \pm .60 | 14.2 \pm .49 | 7.46 \pm .42 |
| | ♀ Dystrophic | 7 | 12.8 \pm .36 | 13.3 \pm .11 | 6.69 \pm .43 |
| | ♀ Non-dystrophic | 6 | 13.9 \pm .36 | 14.0 \pm .12 | 6.88 \pm .20 |
| Kidney | ♂ Dystrophic | 5 | 5.49 \pm .33 | 5.51 \pm .24 | 3.95 \pm .19 |
| | ♂ Non-dystrophic | 6 | 4.39 \pm .12 | 4.51 \pm .11 | 4.41 \pm .47 |
| | ♀ Dystrophic | 7 | 5.33 \pm .22 | 5.44 \pm .17 | 4.41 \pm .45 |
| | ♀ Non-dystrophic | 8 | 4.98 \pm .13 | 5.17 \pm .15 | 4.89 \pm .40 |
| Lung | ♂ Dystrophic | 3 | 6.52 \pm .15 | 6.60 \pm .07 | 7.19 \pm 1.2 |
| | ♂ Non-dystrophic | 4 | 5.77 \pm .10 | 5.90 \pm .10 | 5.63 \pm 1.5 |
| | ♀ Dystrophic | 3 | 6.64 \pm .19 | 6.82 \pm .37 | 5.78 \pm .18 |
| | ♀ Non-dystrophic | 3 | 6.25 \pm .33 | 6.40 \pm .35 | 5.88 \pm .34 |
| Heart | ♂ Dystrophic | 2 | 1.77 | 1.91 | 4.59 |
| | ♂ Non-dystrophic | 2 | 1.65 | 1.78 | 3.57 |
| | ♀ Dystrophic | 2 | 2.11 | 2.25 | 4.62 |
| | ♀ Non-dystrophic | 2 | 1.76 | 1.85 | 4.20 |

* \pm stand. error.

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Effects of Some Steroids on Glycogen Metabolism in Uterus and Skeletal Muscle.* (23756)

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While injections of estrogen result in the deposition of glycogen in the rat uterus(1,2, 3), efforts to produce this response with other steroids such as testosterone, adrenal cortical extracts and progesterone have failed(3,4) with one exception(5). The present study concerns an investigation of (a) ability of certain steroids to elevate uterine glycogen in the spayed rat, (b) effects of combinations of these steroids with estrogen on glycogen deposition and (c) their effects on mobilization of glycogen after epinephrine treatment. Glycogen levels in a representative skeletal muscle were determined concomitantly.

Materials and methods. Virgin female rats weighing 180-220 g were spayed 6-9 days before hormones were administered. In all experiments, the steroids[‡] (except estradiol) were injected subcutaneously in 2 mg doses daily for 3 days just prior to autopsy. Estradiol benzoate (either 0.5 or 50 μ g doses) was injected subcutaneously 48 hours prior to autopsy. Epinephrine hydrochloride (Parke Davis, Co.) was administered intraperitoneally in doses of 50 μ g per 100 g of body weight

1 hour before the rats were sacrificed. Primarily for the study on skeletal muscle glycogen, all rats were fasted 24 hours before autopsy. The rectus femoris muscle and the uterus were removed from anesthetized (Nembutal) rats, rapidly weighed on a torsion balance and digested immediately in hot KOH. The methods used for the digestion and precipitation of the glycogen in these tissues were those previously described(3,6). The anthrone procedure(7) was employed to determine the concentration of glycogen in the tissues and the latter is reported as mg of glucose per 100 g of tissue (wet weight). The data on the uterus are reported in terms of total uterine weight, although most of the glycogen present is in the myometrium(3).

Results. When groups of spayed rats were given injections of the several steroids listed in Table I, the uterine glycogen levels of rats receiving testosterone and desoxycorticosterone were slightly but significantly higher than those of the spayed controls. The inability to demonstrate this effect of testosterone previously(3) was probably due to inadequate hormonal treatment (0.5 mg per day for 2 days). Among the several steroids tested, only testosterone produced a considerable increase in uterine weight similar to that observed in rats after adequate estrogen treatment. Thus, it is possible that the deposition of glycogen in uterine tissue (particu-

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TABLE I. Effects of Steroids on Glycogen Content of Uterus and Skeletal Muscle of Spayed Rats.

| Treatment | No. of animals | Uterine wt (mg) | Glycogen (mg/100 g) | |
|----------------------|----------------|-----------------|---------------------|------------|
| | | | Uterus | Leg muscle |
| None (spayed) | 14 | 116 ± 6* | 78 ± 3 | 364 ± 13 |
| Testosterone | 7 | 256 ± 25† | 129 ± 7† | 574 ± 19† |
| Desoxycorticosterone | 8 | 116 ± 11 | 107 ± 7† | 412 ± 11 |
| Cortisone | 6 | 114 ± 9 | 74 ± 3 | 458 ± 17† |
| Cortisol | 8 | 64 ± 5 | 113 ± 19 | 519 ± 36† |
| 11-Desoxycortisol | 8 | 83 ± 6 | 69 ± 5 | 464 ± 35 |
| Pregnenolone | 5 | 94 ± 17 | 86 ± 5 | 426 ± 16 |

* Mean ± stand. error of mean.

† Mean significantly greater than controls, $P < .01$.

larly in the myometrium) induced by testosterone and estrogen is intimately related to the mechanism by which these hormones exert their anabolic effects, as suggested by previous work(6). The slight elevation in uterine glycogen following injection of desoxycorticosterone cannot be explained similarly since this steroid failed to stimulate uterine growth. However, glycogen storage in the uterus is not necessarily a concomitant of uterine growth, for increases in uterine glycogen have been obtained with doses of relaxin which did not alter uterine weight(8). The failure of the uterus to increase in weight following treatment with the other steroids tested agrees with previous findings(9,10).

The rectus femoris muscles of rats injected with testosterone, cortisone and cortisol contained considerably more glycogen than those of control animals. Pregnenolone increased skeletal muscle glycogen slightly but not significantly at the P value chosen ($< .01$). Previously testosterone and cortisone were shown to increase the glycogen content of skeletal muscle(6,11,12), while cortisol was reported to lack this activity in the gastrocnemius muscle of the normal rat(13). Other than the fact that a different muscle was used, it is difficult to understand why the latter investigators failed to obtain a response with this steroid.

The next study was made to determine the effects of some of these steroids on the deposition of glycogen induced by estradiol. Relatively large amounts of cortisone or cortisol were injected together with a dose of estradiol (0.5 μ g per rat) which significantly increased uterine glycogen without producing a maximum response. It was found that neither of these adrenal cortical steroids significantly af-

fected the deposition of uterine glycogen resulting from the action of estradiol (Table II). Progesterone also fails to modify the effect of estradiol on glycogen deposition in the uterus(4).

These results warrant a reconsideration of the role played by estrogen in the stimulation of uterine glycogen synthesis. It is well known that uteri removed from rats previously treated with estrogen have high rates of aerobic and anaerobic glucose uptake, as measured by the rate of disappearance of glucose from the incubation medium(14-17). The *in vivo* accumulation of glycogen in the uterus after estrogen administration has been considered(15,16) as the direct result of accelerated uptake of glucose from the blood. If this opinion is valid, cortisol, which inhibits the estrogen-stimulated *in vitro* uptake of glucose by the uterus(17), might also impair the *in vivo* accumulation of uterine glycogen. In the present experiment, however, neither cortisol nor cortisone interfered with the deposition of uterine glycogen induced with estradiol. The possi-

TABLE II. Effect of Some Steroids on Deposition and Mobilization of Glycogen in Estradiol-Treated Spayed Rats.

| Treatment | No. of animals | Glycogen (mg/100 g) | |
|---------------------------|----------------|---------------------|------------|
| | | Uterus | Leg muscle |
| Estradiol (.5 μ g) | 7 | 216 ± 19* | 400 ± 27 |
| + cortisone | 9 | 175 ± 11 | 501 ± 31 |
| + cortisol | 6 | 205 ± 9 | 586 ± 50† |
| Estradiol (50 μ g) | 20 | 321 ± 12 | 450 ± 14 |
| + cortisone | 8 | 315 ± 14 | 479 ± 18 |
| + epinephrine | 6 | 161 ± 13† | 294 ± 19 |
| + cortisone & epinephrine | 11 | 73 ± 12† | 323 ± 27 |

* Mean ± stand. error of mean; effect of estradiol can be noted by comparing with spayed controls, Table I.

† Mean significantly different from estradiol-treated controls, $P < .01$.

bility exists that insufficient quantities of the cortical steroids were injected to successfully compete with the amount of estradiol administered, although sufficient amounts were present to stimulate the deposition of glycogen in the rectus femoris muscle (Table II). On the other hand, it is conceivable that interference with uterine glucose uptake (at the glucokinase level) does not seriously impair the deposition of glycogen under estrogen stimulation, since there is no evidence which indicates definitely that blood glucose is the material utilized in the synthesis of uterine glycogen.

An experiment was also performed to determine the influence of cortisone on the glycogenolytic action of epinephrine in the uterus. Spayed rats were given doses of estradiol which elevated uterine glycogen to near maximal levels and a dose of epinephrine was employed which lowered uterine glycogen significantly. Another group of similarly treated spayed rats were given cortisone in addition. The glycogenolytic action of epinephrine in the presence of cortisone was increased markedly and still lower glycogen levels resulted (Table II). Thus, cortisone potentiated the glycogenolytic action of epinephrine in the uterus.

It was previously demonstrated that in skeletal muscle (rectus femoris) cortisone inhibited(12) and desoxycorticosterone potentiated(18) the glycogenolytic action of epinephrine. The glycogen levels of the rectus femoris muscle were also determined in the above experiments and the results indicated that epinephrine decreased the glycogen of the leg muscle to a similar extent in rats either with or without cortisone treatment (Table II). Thus, an inhibition of the glycogenolytic action of epinephrine was not exhibited. However, the dose of epinephrine employed was quite large and probably nullified the inhibiting effect of the cortisone treatment, since previous work(12) stressed the importance of

the proper dose relationship in order to demonstrate this antagonism of epinephrine by cortisone.

Summary. (1) Among a series of steroids which were tested for ability to elevate the glycogen level of the uterus of the spayed rat, testosterone and to a very slight extent, desoxycorticosterone were effective. (2) Cortisol elevated skeletal muscle glycogen, contrary to previous findings. (3) Cortisone and cortisol *in vivo* did not interfere with the deposition of uterine glycogen induced by the injection of small doses of estradiol. (4) Cortisone enhanced the glycogenolytic action of epinephrine in the uterus.

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Metabolic Fate of S^{35} -Labeled Sulfate in Baby Pigs.* (23757)

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Machlin *et al.*(1) reported that a small amount of S^{35} from labeled sulfate is incorporated into the cystine of egg albumin. Some of the sulfur administered in sulfate form to growing chickens was incorporated into taurine, cystine and methionine(2,3). The incorporation of sulfate sulfur into taurine by developing chick embryos was also demonstrated(4). Dziewiatkowski(5) demonstrated incorporation of S^{35} from sulfate into cystine to a small extent by the young rat and pointed out that highest concentration of S^{35} -labeled cystine occurred in the fraction containing the intestinal tract and its contents, suggesting that the bacteria of the intestinal tract were involved in synthesis of labeled cystine. Dziewiatkowski and DiFerrante(6) demonstrated S^{35} -labeled cystine and methionine in sera of rats that had been injected intraperitoneally with S^{35} -labeled sulfate. The conversion of sulfate sulfur into cystine and methionine sulfur by the ruminant has been demonstrated(7,8).

Incorporation of S^{35} from labeled sulfate by the rabbit(9-11) results in highest uptake in cartilaginous tissues. Heath *et al.*(12) reported that when labeled sulfate was fed to a one-year-old boar, a large amount of the S^{35} appeared in urine. The urinary S^{35} level dropped rapidly during the first 2 weeks. Sulfur 35 values in blood, sperm and seminal plasma were also reported. The purpose of the present study was to obtain further information about the metabolic fate of S^{35} sulfate administered to pigs with particular attention to conversion of sulfur into organic forms.

Methods. A single dose containing 1 millicurie of S^{35} -labeled sulfate together with 0.2 mg of carrier sodium sulfate was administered

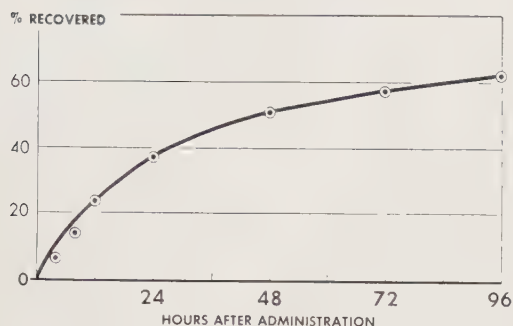
by stomach tube to each of three 17-day-old litter-mate Landrace gilts. The baby pigs were individually caged and fed pasteurized cows' milk 4 times daily for 4 days preceding and during the 4-day collection period. Selected tissues were processed to determine the concentration of S^{35} present(11). Intestinal tract contents were removed, dried at 60°C for 24 hours and then analyzed for S^{35} . In addition, 1 g samples (air-dry basis) of intestinal tract contents from each of the pigs, as well as samples from pig No. 3 of liver, skin, heart and red bone marrow were hydrolyzed by refluxing for 2 hours in a mixture containing 20 ml of 90% formic acid and 40 ml of 6 *N* hydrochloric acid. (Guranani *et al.* 13). The samples were evaporated to dryness under a partial vacuum, below 50°C. Distilled water was added and the samples were again evaporated to dryness. The residue was dissolved in 20 ml of distilled water. A 10 ml aliquot was fractionated by a modification of the procedure described by Mueller *et al.*(14). Resin columns which were 0.9 cm in diameter and 5 cm in height were used. An additional 100 ml of 6 *N* hydrochloric acid after 10 ml were used as indicated in the published procedure. The column was washed with distilled water until the effluent was neutral, and then with an additional 20 ml of distilled water and 40 ml of 1 *N* ammonium hydroxide. When a solution containing sulfate, methionine and cystine was put on the column, all sulfate was eluted by water in the first fraction, methionine was eluted by 0.8 *N* hydrochloric acid in 55% ethanol and cystine by 6 *N* HCl. However, in the case of the hydrolysates of the intestinal tract contents, all of the S^{35} was not eluted by 110 ml of 6 *N* hydrochloric acid, and from 1.2 to 1.5% of the S^{35} was eluted in the final fraction for which 40 ml of 1 *N* ammonium hydroxide was the elutrient. In order to characterize the forms in which the S^{35} occurred in the hydrolysate of the intestinal tract contents, fractionation on a 0.9 x 100 cm Dowex—50 x 12

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URINARY SULFUR 35 EXCRETION

By Baby Pigs Given Labeled Sulfate Orally

FIG. 1. Cumulative urinary S^{35} excretion curve.

columns in the ammonium cycle was carried out with the sample from pig No. 3. An aliquot of the hydrolysate representing 150 mg of dry matter was put on the column. About 64 ml of pH 2.0, 0.1 *M* ammonium formate was used to elute the column, followed by 196 ml of pH 3.4, 0.1 *M* ammonium formate, 194 ml of pH 4.3, 0.1 *M* ammonium acetate, 141 ml of pH 5.0, 0.1 *M* ammonium acetate and 69 ml of 1 *N* ammonium hydroxide. The flow rate ranged from 3.5 to 4.4 ml/hour. The use of pH 2.0, pH 4.3 and pH 5.0 buffers was suggested by the research of Davison(15).

Results. The urinary S^{35} excretion data are presented in Fig. 1. Most of the labeled sulfur was rapidly absorbed and then excreted by the renal pathway. Within the first 4 hours after dosing an average of about 7% of the dose was excreted in the urine. Table I presents data obtained by analysis of intestinal tract contents. Only a small part of the dose, less than 2%, appears in the intestinal tract contents, as compared with about 62% of the dose which was excreted in the urine. Fractionation of hydrolysates of intestinal contents from the 3 pigs by use of the 5 cm high columns as outlined above revealed that from 45 to 63% of the S^{35} present was in organic form. Most of this S^{35} was in the fractions eluted by the 0.8 *N* hydrochloric acid in 55% ethanol and the 6 *N* hydrochloric acid. However, from 1.2 to 1.5% of S^{35} recovered appeared in the final fraction, which was eluted by 1 *N* ammonium hydroxide. From 35 to 55% of S^{35} was in the sulfate-containing first fraction which was eluted by distilled

water. The results of fractionation of S^{35} in the hydrolysate of intestinal tract contents from pig No. 3 by means of the 100 cm resin column revealed a well defined sulfate peak which contained 52% of total S^{35} . This was in general accord with the finding that 51% of total S^{35} from another aliquot of this hydrolysate which was run on the 5 cm resin column was contained in the first fraction, eluted by water. A cystine peak containing about 2.9% and a methionine peak containing about 13.3% of total S^{35} were eluted from the 100 cm resin column by pH 4.3 buffer from the 100 cm resin column. Identity of these peaks was confirmed by paper chromatography. A third peak, unidentified, was eluted by the pH 5.0 buffer and contained about 6.4% of the total S^{35} . In addition, about 14.7% of total S^{35} was eluted by 1 *N* ammonium hydroxide in a fourth peak.

The tissue S^{35} concentration values are presented in Table II. Highest S^{35} uptake occurred in ear cartilage. This is similar to findings in studies in which labeled sulfate was administered to rabbits(9-11). The fractionations by means of ion exchange revealed that almost all tissue S^{35} was in the sulfate-containing fraction. In the case of the hydrolysates of heart and red bone marrow samples, no organic- S^{35} was detected. About 0.01% of organic- S^{35} was detected in skin hydrolysates and 1.45% in liver hydrolysates.

The finding that a large part of the S^{35} in the intestinal tract contents had been converted to organic form while only a small amount of organic- S^{35} could be detected in tissue samples suggests that the microbial population of the intestinal tract was probably responsible for the incorporation of S^{35}

TABLE I. Intestinal Tract Contents of Baby Pigs 4 Days after Oral Administration of Labeled Sulfate.

| Pig No. | 1 | 2 | 3 | Avg |
|--|------|------|------|------|
| Wt (g) | 8.4 | 10.0 | 4.9 | 7.8 |
| % dose/g | .15 | .19 | .16 | .17 |
| % dose in total | 1.25 | 1.90 | .78 | 1.31 |
| % of S^{35} in sulfate-containing fraction | 55.2 | 36.6 | 50.7 | 47.5 |
| % of S^{35} as in organic sulfur fraction | 44.8 | 63.4 | 49.3 | 52.5 |

TABLE II. Tissue S³⁵ Concentration in 3-Week-Old Pigs 4 Days after Oral Administration of Labeled Sulfate.

| Tissue | Pig 7 (wt 3995 g) | | Pig 2 (3445 g) | | Pig 3 (4035 g) | | Avg (3825 g) | |
|-------------------------|--------------------------------|--------------------|--------------------------------|--------------------|--------------------------------|--------------------|-------------------------------|--------------------|
| | % dose*/g × 10 ³ | % dose in organ | % dose*/g × 10 ³ | % dose in organ | % dose*/g × 10 ³ | % dose in organ | % dose/g × 10 ³ | % dose in organ |
| Liver | 4.50 | .47 | 2.84 | .29 | 4.12 | .38 | 3.82 | .38 |
| Kidneys | 3.75 | .07 | 2.29 | .05 | 2.55 | .06 | 2.86 | .06 |
| Heart | 2.03 | .04 | 1.52 | .03 | 1.45 | .03 | 1.67 | .03 |
| Gastrocnemius muscle | 1.65 | | .81 | | 1.13 | | 1.20 | |
| Ear cartilage | 83.50 | | 47.64 | | 61.92 | | 64.35 | |
| Skin | 8.89 | | 6.54 | | 6.87 | | 7.43 | |
| Brain | 3.01 | | 2.18 | | 2.61 | | 2.93 | |
| Red bone mar- row | 46.86 | | 39.71 | | 43.27 | | 43.29 | |
| Femur shaft | 13.14 | | 9.41 | | 8.95 | | 10.50 | |
| Aorta | 24.27 | | 16.80 | | 19.30 | | 20.12 | |
| Serum | 4.10 | | 3.31 | | 4.20 | | 3.87 | |

* Values standardized to body wt of 3700 g.

into organic form. Dziewiatkowski's research with the rat(5) suggests that a similar situation exists with this species.

Summary. The metabolic fate of S³⁵ administered by stomach tube as sulfate to baby pigs has been studied. About 62% of the dose was excreted in urine within 4 days. Of tissues analyzed, ear cartilage, red bone marrow and aorta showed highest concentrations of S³⁵, and brain and muscle lowest concentrations, 4 days following dosing. About half of the S³⁵ in intestinal tract contents was in organic form, while only very small amounts of organic-S³⁵ were detected in tissues examined.

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Transplacental Infection of Fetuses of Rabbits With Herpes Simplex Virus. (23758)

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There is increasing evidence of the role of the blood in dissemination of a virus from the portal of entry to the sites of infection in various organs(1) and in the fetuses. Belenky (2) reported the presence of vaccinia virus in the blood and placenta of pregnant animals following vaccination but was unable to detect the virus in the amniotic fluid, umbilical cord or in the organs and blood of the embryos. Berry and Slavin(3) were able to isolate herpes simplex virus from the blood of infected adult mice but were not able to isolate the virus from their embryos. Scheidegger(4) reported that the embryos of mice died when the adults were inoculated with psittacosis or ectromelia viruses. Swan(5), Gregg (6) and others have described cases of congenital malformations of children born to mothers who have been infected during pregnancy with rubella virus. Winsser, *et al.*(7) discovered a viremia in a newborn whose mother suffered paralytic poliomyelitis shortly before childbirth and Schaeffer, *et al.*(8) isolated the poliovirus from the aborted fetus of a mother who had the disease.

Our report describes studies initiated to determine if herpes simplex virus crosses the placental barrier of an infected, pregnant rabbit during the viremia stage and invades the fetal tissues.

Materials and methods. The HF strain of herpes simplex virus obtained from the Communicable Disease Center, Montgomery, Ala., and propagated in this laboratory on the chorioallantoic membranes (CAM) and in the yolk sacs of embryonated hen's eggs, in the brains of white Swiss mice and on the cornea of the rabbit was used as the infecting agent. Five young adult white doe rabbits were bred with bucks of the same strain. Fourteen days

after breeding, the does were lightly anesthetized with ether and 0.1 ml of the virus suspension was massaged into the scarified cornea. One ml of blood was removed aseptically from the marginal ear veins of the rabbits at 24 hours and 48 hours and every 12 hours thereafter through 180 hours. Blood samples were also taken just prior to surgery. The presence of the virus in these samples was determined by the production of lesions on the CAM(1). Fifty-four hours following inoculation, the rabbits were anesthetized with ether and surgical procedures were used to remove one gravid horn of the bifurcate uterus. The other horn was left intact so that the rabbit might go to term and deliver. The newborn was then examined for any fetal anomalies that might be present. The fetuses from each rabbit were dissected from the excised uterine horns, washed thoroughly with sterile physiological saline and ground in sterile tissue grinders. This material was diluted 1:2 with buffered gelatin saline and 0.1 ml of this homogenate of fetuses from each rabbit was inoculated onto each of 8 CAM. The inoculated chick embryos were incubated for 72 hours. At this time the membranes were harvested and examined for typical herpes simplex plaques which would indicate the presence of the virus in fetal tissue.

Results. Viral isolations were accomplished from the blood specimens taken at 24 hours, 48 hours and at the time of surgery from all 5 rabbits. The virus was also demonstrated from blood specimens at varying times through 156 hours. All of the CAM inoculated with homogenates obtained from the fetuses of all 5 of the experimental rabbits showed the presence of typical herpetic plaques, indicating the presence of herpes simplex virus in the fetal tissue. Comparative titrations of the virus isolated from the fetuses were performed according to Scott(9) in the presence of specific immune serum and buffered gelatin saline in order to identify the

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TABLE I. Results of Neutralization Test on CAM Using Virus Recovered from Fetuses of Rabbits Infected with Herpes Simplex Virus.

| Inoculum | Dilutions of the virus | | | | | | | |
|-------------------|------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | 10 ⁻⁰ | 10 ⁻¹ | 10 ⁻² | 10 ⁻³ | 10 ⁻⁴ | 10 ⁻⁵ | 10 ⁻⁶ | 10 ⁻⁷ |
| Virus + antiserum | 4+* | 4+ | 3+ | 2+ | 2+ | + | — | — |
| Virus + BGS | 4+ | 4+ | 4+ | 3+ | 3+ | 3+ | 2+ | + |

* Plaque formation on CAM.

4+, membranes heavily infected; 3+, membranes contained 16-25 plaques; 2+, membranes contained 6-15 plaques; +, membranes contained 1-5 plaques; —, membranes contained no plaques.

CAM, chorioallantoic membranes; BGS, buffered gelatin saline.

isolated virus. Representative results (Table I) demonstrated that infectivity of the virus was decreased by the specific immune serum.

Discussion. Herpes simplex virus was recovered from the fetuses of the 5 experimentally infected rabbits by inoculation of fetal homogenate on the CAM of the developing chick embryo. The virus was proved to be herpes simplex by the neutralization of infectivity with specific immune serum.

The finding of herpes simplex virus in the blood of these experimentally infected animals confirmed previous results(1) and thus indicated that the fetuses were probably infected by the virus crossing the placental barrier.

Zuelzer and Stulberg(10) presented data indicative of a viremia stage in newborn infants who succumbed to overwhelming herpetic infections. With the results here reported, the role of a viremia stage in the infection of fetuses of a herpes simplex infected rabbit could prove to be a valuable lead in discovering other possible causes of congenital malformations in newborn infants.

These findings pose several questions: What role does this virus play in causing fetal anomalies? Can this virus serve as the primary infectious agent and remain in a latent state in the offsprings' tissues to mani-

fest itself later in life? If the virus can thus invade fetal tissues early in life, what role does it play in failure of an animal to build up protective antibodies against future exposure to this virus? The answers to these and other questions are now being sought.

Summary. Herpes simplex virus was isolated from the fetuses of 5 rabbits which had been inoculated intracorneally with herpes simplex virus. The isolated virus was identified with specific immune serum.

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Effects of Reserpine and Chlorpromazine upon Enterochromaffin Cell of the Rat. (23759)

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(Introduced by Chauncey D. Leake)

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As part of an effort to study the effects of drugs upon specific cell types, a program that we call "histopharmacology," we have been interested in examining certain drug-cell systems as models for future development of this field. One of these that has been studied is the reserpine-enterochromaffin cell interaction. Serotonin (5-hydroxytryptamine) is considered from histochemical evidence to be the storage and secretion product of the enterochromaffin cells, which are located in the gastrointestinal mucosa of all vertebrates except the cyclostomes and teleosts(4). Concentration of serotonin decreased in the intestine after administration of reserpine, one of the Rauwolfia alkaloids with tranquilizing action(7). The decrease was found to occur only with this group of alkaloids possessing a sedative action(2). Chlorpromazine, a synthetic, psychotherapeutic drug also exerting sedative action, was shown to be incapable of decreasing serotonin concentration and exhibits what is thought to be a different mechanism of action(3). Recently, Benditt and Wong(1) described an effect of reserpine upon enterochromaffin cells of the guinea pig, finding that these cells diminish in number following exposure to the drug. Feeling that the change in absolute number of cells per unit area is too crude an estimation of cytological response, we examined the effects of reserpine in producing changes in the most important cytological characteristic of these cells, namely the granulation which could be revealed by silver staining. Chlorpromazine was used as a pharmacological control.

Materials and methods. Observations were made upon microscopic sections of the duodenum of 5 series of drug-injected and saline-injected male albino rats, comprising a total of 30 animals. Eighteen of these were used in the reserpine study, divided into experimental and control series. The experimental and control animals were matched by weight.

The experimentals were injected intraperitoneally with 5 mg/kg of reserpine, and the controls were simultaneously treated with the same volume per body weight of physiological saline as in the reserpine-injected series. Reserpine-injected and saline control animals were sacrificed at 8, 16 and 24 hours after treatment. In the chlorpromazine study injections were made in the same manner as in the reserpine-injected series, using 12 animals. Sections of duodenum 2-3 cm in length, adjacent to and including a small part of the pylorus, served as the source of the enterochromaffin cells whose responses were studied. Tissues were washed with physiological saline and fixed immediately in a 10% aqueous formalin solution. After 3 days of fixation tissues were infiltrated and embedded in a special embedding mass, M.P. 58-65°C. Longitudinal sections (1.0 cm) were cut at 6 μ . Of 4 different methods employed, the methenamine silver technic of Gomori(5) was found to be the most reliable for specifically differentiating enterochromaffin cells, and revealing cellular detail. All sections used for analysis were reacted under the same conditions at the same time (60°C for 3-3½ hours, buffered at pH 7.8 with Holmes' alkaline borate buffer prior to impregnation). In attempting to establish a quantitative method for arriving at total granulation in the duodenum an arbitrary numerical assay or *weighted granulation count* was established. Cells were classified into 4 groups according to the density or apparent number of granules, from 1 plus (+), the least dense, to 4 plus (++++), the most dense (Fig. 1). The enterochromaffin cells in 4 random sections, each 1.0 cm in length, were counted for each experimental and control animal. The *weighted granulation count* was achieved by multiplying the total number of cells in each granulation stage by the numbers 1, 2, 3 and 4, corresponding to the differences in total granulation, as es-



FIG. 1. Density classification of enterochromaffin cells of rat duodenum. Methenamine-silver stain.

established previously. Thus, the number of plus 1 was multiplied by 1, the number of plus 2 was multiplied by 2, etc. This is a conservative adaptation of a weighting device used by the Hartrofts in their study of the renal juxtaglomerular granulation(6). They used the geometric series 1, 2, 4 and 8 to weight the granulation index. It is felt that the arithmetic series used in this study more accurately represents the quantitative differences in the granule content of the cells studied. These *weighted granulation counts* were totaled, and the average count was calculated for a 1.0 cm section of duodenum. This figure was expressed as the *Weighted Granulation Index*. In presenting experimental results the *Weighted Granulation Index* of each animal was expressed as percentage difference of control minus the experimental. Fig. 2 shows the percentage differences in weighted granulation indices for the reserpine and chlorpromazine series, sacrificed at the different time intervals.

Results. It was found that an average 1.0 cm section of rat duodenum contained 183 enterochromaffin cells, predominantly of the moderately granulated types. Weighted granulation indices averaging 457 were obtained for controls. There was a variation of cell count with the weight of the animal, so that it was important to match the weights of the controls and the experimentals quite

closely. Intraperitoneal injection of reserpine caused a decrease in the total enterochromaffin cell granulation at 8 and 16 hours, and a marked increase at 24 hours to an apparently significant degree in the rat. The changes were primarily due to changes in the degree of granulation rather than a change in the absolute number of cells. Chlorpromazine was found to have no significant change upon enterochromaffin cell granulation at 8, 16 and 24 hours. Pharmacological and physiological effects of both drugs were noted and were found to be in agreement with observations reported by other authors.

Discussion. The pharmacologic relationship of reserpine action to serotonin release, as developed by Brodie's laboratory, has been discussed(8,9). These results have been based upon chemical estimation of serotonin in tissue or serotonin metabolite in urine. Our findings on the changes in the enterochromaffin cell granulation parallel intestinal serotonin assay closely, in that both show a minimum at 16 hours. However, where the chemical values do not return to normal for several days, the cellular pattern has been restored at

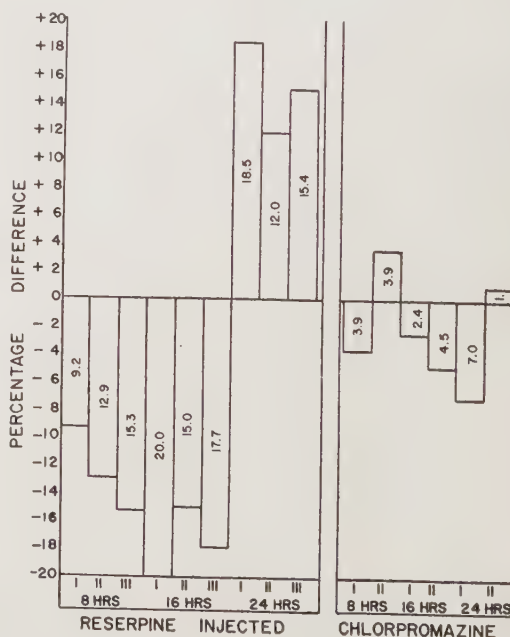


FIG. 2. Response of enterochromaffin cells to reserpine and chlorpromazine. Percentage difference in weighted granulation indices of treated and control rats.

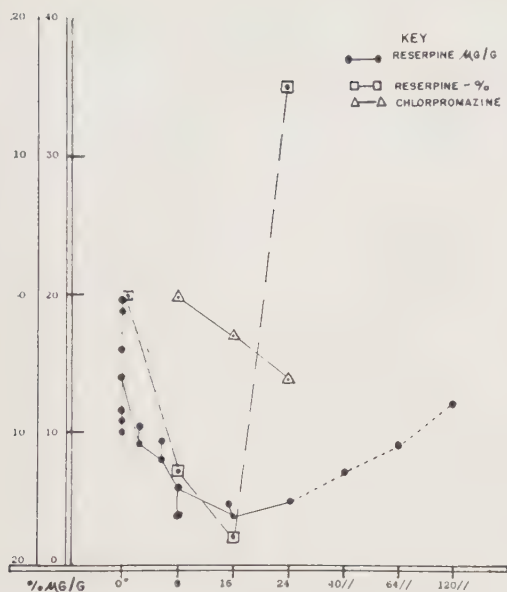


FIG. 3. Comparison between histological and chemical estimate of reserpine effect upon the enterochromaffin cell. The 2 ordinates are percent change of weighted granulation indices and μg of serotonin of intestine. The latter data are from Pletscher(7).

24 hours (Fig. 3). It is believed that this represents a period of resynthesis and reaccumulation of serotonin, or a precursor, without release. Cytological data are not sufficient to confirm this. Benditt and Wong(1), describe a similar effect upon enterochromaffin cells to that noted here. We feel that their method, that of counting cells per unit area and ignoring the intensity of granulation, does not offer the degree of precision required for pharmacological evaluation. On the other hand, weighting each cell according to its degree of granulation does give a better esti-

mate of graded change along with a dynamic picture of cytological response to drug action. Also we feel it important that the experimental and control animals be matched by weight as our data indicate that the cell count and the weight or age may be correlated, perhaps due to a change in thickness of the mucous membrane with age.

Summary. Cytological evidence has been obtained, using a semiquantitative technic, for the action of reserpine upon enterochromaffin cells. Concomitant with release of serotonin, there is a loss of granulation in these cells. Chlorpromazine, as a pharmacological control, was found to be without significant cytological effect. The method of the *Weighted Granulation Index* offers one approach to the quantitative study of drug effects upon specific cell types.

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Plasma Volume and Electrolyte Changes Induced by 2-Methyl-9 Alpha Fluorohydrocortisone in Fasted Adrenalectomized Dogs.* (23760)

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Hormones of the adrenal cortex appear to exert a profound regulatory control over the internal distribution of water and certain electrolytes(1-2). There are clear indications of fluid, Na, Cl and K shifts between intra- and extracellular body compartments following i.v. administration of potent analogs of hydrocortisone such as 2-methyl-9 α -fluorohydrocortisone (2-methyl FF) and 1-dehydrohydrocortisone (1-dehydro F), to fasted adrenalectomized dogs recovering from severe insufficiency. Further evidence supporting this view of adrenal cortical function has been obtained by simultaneous determinations of plasma volume and electrolyte changes in healthy adrenalectomized dogs receiving the potent corticoid 2-methyl FF while deprived of food and water.

Methods. Sixteen adrenalectomized dogs were used; in the interval between experiments they were maintained in good health by daily i.m. injections of 0.5 mg DCA in oil, plus 2 g of salt supplements in the food. The extra salt was withheld 3 days before an experiment and the dogs were fed a ration which allowed them a total daily intake of 1.47 g of Na and 0.94 g of K. Twenty-four hours after the last feeding, blood samples were taken, the bladder drained by catheter and the animals placed in metabolism cages. Food and water were then withheld for 48 hours. The control data obtained are designated "initial" on Table I. The dogs received no food for 24 hours before sampling but had access to water hence they were 72 hours without food at termination of the experi-

ment. Intravenous injections of 2-methyl FF were administered daily in divided doses (Tables I and II). Free alcohol of 2-methyl FF was injected in 25% absolute ethanol, 25% propylene glycol and 50% water. The compound was dissolved in hot alcohol and with constant stirring the hot propylene glycol was added dropwise. This was followed by hot water delivered in the same manner. These procedures were necessary because of the relative insolubility of the compound in an aqueous medium. The method for plasma volume determination was essentially that of Chinard(3), using a standardized sample of the dye T-1824. After withdrawal of a dye-free blood sample from the jugular vein employing a heparinized syringe, a measured sample of the dye was injected into the jugular from an accurately calibrated syringe, the syringe being cleared of all dye by rinsing repeatedly with blood. Samples were collected from this vein without stasis through an in-dwelling needle at 10, 13, 16 and 19 minutes after injection. In order to account for any residual dye which might be retained from the previous determination, the initial sample, taken before dye injection, was used to set the zero on the Beckman Model B spectrophotometer employed for the determinations. All values for plasma volume were calculated from semi-log plots(4). Quantity of blood withdrawn for electrolyte and plasma volume study was 37 cc for each of the 3 samples, or a total of 111 cc removed over 48 hours. The plasma loss was compensated for by i.v. injections of an equivalent amount of 5.5% glucose, calculated from the hematocrit. Two "control" dogs were simply continued on the routine maintenance therapy of daily i.m. injections of 0.5 mg DCA without other steroid treatment during the fasting period. Each of these dogs was also injected twice daily i.v. with 7.5 cc of the vehicle used for solubilizing the 2-methyl FF.

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TABLE I. Plasma Volume and Hemoconcentration Changes Induced in Fasted Adrenalectomized Dogs by 2-Methyl FF.

| No. of dogs averaged | Dose, mg/day | Hr bled | Body wt, kg | BP, mm Hg | Blood urea N, mg % | Hb, g % | Hmet, % | RBC 10 ⁶ , mm ³ | Blood sugar, mg % | Plasma vol, cc/kg |
|--|--------------|---------|-------------|-----------|--------------------|---------|---------|---------------------------------------|-------------------|-------------------|
| 2-Methyl FF (free alcohol) | | | | | | | | | | |
| 5 | | Initial | 14.8 | 114 | 11.0 | 12.9 | 35.0 | 4.67 | 80 | 47.1 |
| | 1 | 24 | 14.1 | 117 | 10.1 | 11.2 | 31.6 | 3.85 | 86 | 55.2 |
| | 1 | 48 | 13.7 | 113 | 8.8 | 10.1 | 29.3 | 3.26 | 90 | 62.7 |
| 2 | | Initial | 20.8 | 114 | 18.2 | 11.8 | 32.8 | 5.47 | 80 | 48.5 |
| | 3 | 24 | 19.6 | 114 | 10.9 | 9.9 | 26.9 | 4.59 | 78 | 64.5 |
| | 3 | 48 | 19.0 | 98 | 9.4 | 9.0 | 27.7 | 3.38 | 85 | 66.5 |
| 2 | | Initial | 18.7 | 99 | 22.5 | 13.4 | 34.4 | 5.12 | 77 | 60.5 |
| | 4.5 | 24 | 17.6 | 107 | 19.1 | 11.1 | 31.7 | 4.04 | 85 | 70.8 |
| | 3 | 48 | 16.9 | 102 | 13.4 | 9.6 | 29.5 | 3.57 | 82 | 73.5 |
| 3 | | Initial | 14.4 | 113 | 20.6 | 12.2 | 34.4 | 4.78 | 86 | 59.0 |
| | 5 | 24 | 14.1 | 111 | 14.8 | 10.7 | 29.4 | 4.14 | 95 | 50.0 |
| | 5 | 48 | 13.6 | 121 | 13.1 | 10.5 | 29.4 | 4.22 | 96 | 63.0 |
| 2 | | Initial | 12.7 | 108 | 16.6 | 15.5 | 39.8 | 5.63 | 85 | 40.1 |
| | 8 | 24 | 11.7 | 93 | 12.2 | 12.1 | 33.4 | 4.87 | 95 | 47.4 |
| | 8 | 48 | 11.3 | 93 | 14.4 | 10.4 | 32.2 | 3.99 | 103 | 53.5 |
| Desoxycorticosterone acetate-maintained "controls" | | | | | | | | | | |
| 2 | | Initial | 15.1 | 107 | 18.8 | 10.3 | 29.8 | 4.24 | 81 | 50.5 |
| | .5 | 24 | 14.4 | 115 | 19.3 | 10.7 | 28.3 | 5.03 | 70 | 45.2 |
| | .5 | 48 | 14.0 | 105 | 17.3 | 10.1 | 27.8 | 4.62 | 72 | 47.5 |

Hematocrits of dogs were determined by placing the blood in capillary tubes in an air turbine rotated at 20,000 g for 5 minutes, and plasma and urine Na and K concentrations were measured by the Perkin-Elmer flame photometer, Model C. It should be emphasized that the animals used in the experiments were healthy, vigorous dogs which showed no signs of adrenal insufficiency either before or

after completion of these studies.

Results. The data regarding mobilization and internal redistribution of electrolytes and water in the fasted dogs following i.v. injections of 2-methyl FF, are presented in Tables I and II. All of the figures in Table I represent averages of data from animals grouped according to dosage of steroid they received. The dogs lost between 0.8 and 1.8 kg in

TABLE II. Correlation of Na, Cl and K Excretion with Plasma Volume and Plasma Electrolyte Changes in 14 Adrenalectomized Dogs Receiving 2-Methyl FF during a 48-Hour Fast.

| Total dosage 48 hr, mg | Max plasma electrolyte changes, meq/l | | | Total urine electrolyte excr., meq/48 hr | | | Max plasma vol changes | | Total urine vol, cc/48 hr |
|--|---------------------------------------|------|-------|--|-------|-------|------------------------|-------|---------------------------|
| | Na | Cl | K | Na | Cl | K | cc/kg | % | |
| 2 | + 6.3 | +2.8 | - .88 | 17.20 | 28.94 | 18.82 | +19.2 | +50.3 | 440 |
| 2 | +13.0 | +6.2 | - .49 | 6.07 | 29.00 | 27.20 | +23.5 | +52.9 | 325 |
| 2 | + 2.5 | +1.0 | -1.19 | 11.82 | 24.23 | 23.93 | + 6.5 | +12.1 | 530 |
| 2 | + 6.5 | +2.0 | -1.28 | 23.86 | 38.37 | 28.71 | +17.3 | +32.4 | 760 |
| 2 | + 4.5 | -4.1 | -1.30 | 4.85 | 17.72 | 18.30 | +11.1 | +24.3 | 335 |
| 6 | + 5.5 | +3.5 | -1.18 | 7.16 | 41.67 | 10.70 | +23.0 | +44.2 | 355 |
| 6 | + 5.0 | +4.0 | -1.46 | 28.67 | 33.46 | 44.97 | +15.0 | +33.3 | 1050 |
| 7.5 | +13.2 | +6.5 | -1.06 | 18.93 | 28.89 | 39.83 | +14.8 | +23.9 | 515 |
| 7.5 | + 7.5 | +2.8 | - .45 | 21.35 | 45.00 | 33.77 | +26.2 | +44.2 | 1125 |
| 10 | +11.5 | +4.0 | - .43 | 27.92 | 40.19 | 15.21 | not determined | | 370 |
| 10 | +11.0 | +8.1 | - .57 | 53.99 | 64.39 | 23.62 | " | " | 671 |
| 10 | + 7.5 | +1.0 | - .60 | 9.35 | 21.48 | 17.93 | + 4.0 | + 6.8 | 325 |
| 16 | + 6.0 | -2.9 | - .90 | 5.49 | 11.17 | 19.49 | +14.4 | +40.4 | 403 |
| 16 | + 4.0 | -4.9 | - .88 | 6.48 | 10.42 | 26.05 | +12.4 | +27.8 | 428 |
| Desoxycorticosterone acetate-maintained "controls" | | | | | | | | | |
| 1.0 | - .5 | -2.4 | + .06 | 41.92 | 38.12 | 15.16 | - 4.1 | - 7.9 | 567 |
| 1.0 | - 3.5 | -3.6 | + .74 | 42.78 | 35.18 | 16.83 | - 6.4 | -12.9 | 288 |

+ = Increase in electrolyte or plasma vol.

- = Decrease in electrolyte or plasma vol.

weight during the experimental period. The blood urea nitrogen was sharply reduced and the hemoglobin changes indicated marked blood dilution. The initial hemocrits are low due in part to the anemia characteristic of long-term adrenalectomized dogs. However, following injection of the steroid, the packed volume of red cells showed a decrease from the control level. Likewise the reduction in erythrocyte count furnished further evidence that extravascular fluids were entering the blood. Blood glucose increased appreciably during the fast, no doubt owing to the strong glucocorticoid effect of 2-methyl FF. Plasma volume increased by 24-53% in 10 of the 12 steroid-treated dogs in which it was measured. The 2 remaining dogs showed smaller rises of about 7 and 12%. There was, curiously, no suggestion of dose-response effects within the dose range used. The elevation of plasma volume was accompanied in all cases by simultaneous increases in plasma Na concentration. Chloride concentration changes were of lesser magnitude and sometimes in the opposite direction than those of sodium. Plasma K concentration invariably declined (Table II). Electrolyte and plasma volume changes in the 2 fasted control animals continued only on maintenance doses of DCA, were the reverse of those following administration of 2-methyl FF. The plasma volume declined approximately 8-13% but the plasma Na and Cl concentration failed to rise. Blood sugar showed a moderate decline during the fast, as was anticipated, since DCA lacks glycogenic activity.

Discussion. Elevation of the plasma volume was associated with a well defined rise in plasma Na and to a lesser extent with that of Cl. A fall in Na and Cl should occur in the face of an increasing volume of fluid in circulation unless additional electrolytes from some extravascular depot were entering the blood stream. Evidently the steroid induced an outflux of Na and Cl from some as yet ill-defined source for despite expansion of plasma volume, the rise in plasma Na for example, ranged from 2.5 to 13 mEq/l in starved dogs whose plasma volume simultaneously increased 6.8 to 52.9% above control values (Table II). The excess fluid

shifted into the blood presumably came from intracellular sources since exogenous supplies were not available. The "extra" Na which appeared in the plasma and urine of the hormone-treated dog can probably be accounted for by transfer of the cation from the mass of body cells which are known to contain small quantities and perhaps especially from bone and collagenous tissues both of which are considered to be rich in labile Na(5-7). The hormone seems to be particularly active in inducing shifts of intracellular K to the extracellular (and intravascular) compartment and thence to the kidney for elimination. The K diuresis resulting from injection of 2-methyl FF appears to be more pronounced in fasted adrenalectomized dogs recovering from insufficiency than in healthy animals adequately maintained on DCA(1-2). Apparently an undue accumulation of K collects within the cells in the absence of cortical hormones, which the dog is unable to release for renal excretion until the hormone deficit is corrected. Relief of symptoms is closely associated with and apparently dependent upon outflux of water from the cells.

Summary. Adrenalectomized dogs maintained in good health on 0.5 mg/DCA/day were deprived of food and water for 48 hours and repeatedly injected with the analog of hydrocortisone, 2-methyl-9 α -fluorohydrocortisone. Following injection simultaneous increases of plasma volume and plasma Na occurred along with hemodilution and decrease in plasma K and blood urea nitrogen. The animals continued to excrete Na, Cl and K during the fast, leading to further loss of water and electrolyte. The hormone apparently induced an outflux of fluid and electrolyte from unidentified sources but which are presumed to be cells, bone and collagenous tissues. The experiments afford further evidence that the adrenal cortex elaborates hormones which exert regulatory control over internal distribution of water and certain electrolytes between fluid compartments of the body.

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Potassium Acetate Inhibition of *Lactobacillus casei*. II. Strain Differences. (23761)

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It was reported in the first paper of this series(1) that *Lactobacillus casei* 7469 is markedly inhibited by the dual but not separate action of potassium and acetate ions. The inhibition was completely reversed by either lithium or sodium ions and by various fatty acids(1). Occasional failures of potassium acetate to inhibit *L. casei* growth were encountered during the course of this work but were not reported(1) because it appeared then that they were due merely to technical difficulties. It has now been found, however, that strains of *L. casei* differing markedly among themselves in sensitivity to potassium acetate are derivable from our stock culture, and it seems likely on the basis of these findings that our earlier variable results were caused by fortuitous population differences in the experimental inocula.

Methods. The stock culture of *L. casei* 7469 employed in these studies has been maintained in this laboratory since 1943 as described previously(2), with the exception that since 1948 fresh cultures have been prepared only every 3 weeks (originally this was done every 2 weeks). The possibility of accidental contamination during this period was kept at a minimum by preparing 2 or more stock cultures at each transfer time. Successive stock transfers were made only from the original, unused culture in each case, whereas

transfers to experimental media were made exclusively from duplicates of this culture. The stock culture was never purified because it seemed likely that frequent and random selection of single cells for this purpose might lead to early divergence of originally identical strains in different laboratories. The *population characteristics* of this *L. casei* culture with respect to potassium acetate inhibition were determined as follows. Cell suspensions prepared by transferring growth from the stock culture (using a sterile wire) to sterile saline solution were diluted appropriately and plated out in the same medium as that used in maintaining the stock culture (Yeast Dextrose Agar-Difco). The plates were incubated 40 hours at 35° under carbon dioxide, and growth from isolated colonies was transferred either directly to liquid inoculum medium or (by stab) to Yeast Dextrose Agar. Inocula prepared either directly from colony growth or from the stab cultures derived from such growth were tested by the procedures employed previously(1).

Results. Screening of approximately 1000 freshly isolated cultures in several independent experiments over a 3 months period revealed that from 6% to 45% of the isolates in a given series were resistant to inhibition by potassium acetate. The remaining isolates were not only highly sensitive to potassium acetate, but failed to grow normally even at the usual relatively low levels of potassium and acetate unless provided with a supplement of DL-lactate (the effects of sodium and lithium ion(3) were not determined in

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TABLE I. Concentrations of DL-Lactate (C_3) and DL- α -Hydroxycaprylate (C_8) Yielding Half Maximal Response with Different Strains of *L. casei* in Media with Different Potassium and Acetate Concentrations.*

| Strain No. | Medium 1 | | Medium 2 | | Medium 3 | |
|------------|----------|-------|----------|-------|----------|-------|
| | C_3 | C_8 | C_3 | C_8 | C_3 | C_8 |
| 686-21 | 85 | 31 | 186 | 68 | 324 | >102 |
| 22 | 81 | 23 | 186 | 68 | 269 | " |
| 25 | 96 | 28 | 186 | 76 | 251 | " |
| 27 | 96 | 35 | 186 | 96 | 349 | " |
| 29 | 101 | 32 | 186 | 71 | 251 | " |
| 30 | 85 | 28 | 166 | 68 | 288 | " |
| 31 | 71 | 20 | 138 | 59 | 224 | " |
| 33 | 76 | 20 | 178 | 50 | 209 | " |
| 53 | 76 | 23 | 155 | 59 | 178 | " |
| 55 | 96 | 32 | 186 | 81 | 339 | " |

* Media 1-3 were the same as the medium used previously(1) except for variations in potassium and acetate content. Potassium ion and acetate ion concentrations in the 3 media, respectively, were: Medium 1, 9.4 and 146 μ moles/ml; Medium 2, 80 and 146 μ moles/ml, and Medium 3, 130 and 296 μ moles/ml. Medium 1 contained more sodium ion by 70.6 μ moles/ml than media 2 and 3.

these experiments, and acids other than DL-lactic acid were not extensively investigated). The amounts of either DL-lactate or DL- α -hydroxycaprylate required to effect half maximal growth of ten typical sensitive strains in media containing different concentrations of potassium and acetate ions are listed in Table I. It may be seen (Table I) that the inhibitory effects of potassium and acetate on these strains were reversed by elevating the supply of α -hydroxy acid.

Since it had now become evident that potassium acetate resistant forms abounded in the experimental inocula used previously (1), it seemed likely that their development was seriously impeded in the presence of the potassium acetate sensitive forms. This conclusion was verified experimentally by introducing varying numbers of cells of a typical resistant strain (strain 686-35) into a test medium with and without the addition of a constant number of cells of a typical sensitive strain (strain 686-30) with the results shown in Fig. 1. It may be seen (Fig. 1) that in the test medium containing 6×10^6 cells of strain 686-30 per ml, 40 hour growth of strain 686-35 inocula of less than 4×10^5 cells per ml was markedly inhibited. When potassium acetate was added to the test medium this effect was

augmented as shown in Fig. 2. In this case it may be seen (Fig. 2) that 40 hour growth of strain 686-35 from an inoculum of 8×10^5 cells per ml was reduced from maximal to nearly negligible by the presence of 6×10^6 cells of strain 686-30 per ml.

It is evident from the foregoing results that growth of the sensitive and resistant strains is markedly interdependent in α -hydroxy acid-free media. The former organisms depend on the latter to produce growth promoting material,[†] but under unfavorable conditions growth of the latter is inhibited by the presence of the former. A reasonable hypothesis based on these considerations is that

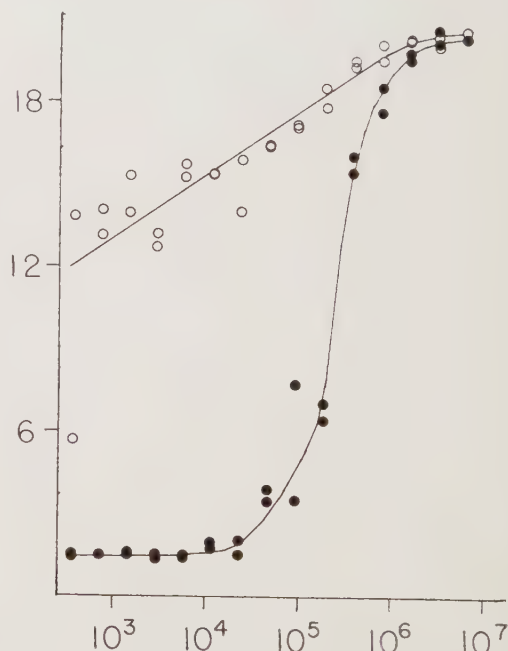


FIG. 1. Production of acid by graded inocula of strain 686-35 after 40 hr incubation in Medium 2 (see Table I) alone (O) and with the addition of 6×10^6 cells of strain 686-30/ml (●). Values on vertical scale represent acidity calculated as ml of 0.01 N sodium hydroxide required to titrate 1 ml of culture. Values on horizontal scale represent the numbers of cells of strain 686-35 introduced per ml.

[†] That *L. casei* normally produces a substance with hydroxy acid-like growth promoting activity has been recognized for several years(4). This substance was originally thought to be D-lactic acid, but it is now known that D-lactic acid is not formed in measurable amounts by *L. casei* and that the growth promoting substance is a more lipophylic acid(5).

the sensitive strains elaborate toxic material when incubated in α -hydroxy acid-free media but not when a source of this growth promoting material is provided. Potassium acetate inhibition of mixed cultures, according to this hypothesis, could result from the increased α -hydroxy acid demand of the sensitive strains in high potassium acetate media (Table I). It also seemed possible that production of growth promoting material by the resistant strains might be impeded at elevated potassium acetate concentrations. The latter hypothesis was tested by culturing three freshly isolated resistant strains (strains 697-7, 697-16 and 697-32) in a) Medium 1 (Table I), b) Medium 3 (Table I), and c) Medium 3 with the addition of 10 m μ mol of linoleic acid per ml. The 40 hour cultures were clarified by centrifugation and assayed for growth promoting activity with strain 280-16A(6). The results shown in Table II indicate not only an appreciable inhibitory effect of potassium acetate on production of growth promoting activity (growth and total acid production are apparently not affected),

TABLE II. Growth Promoting Activity Produced by Different Strains of *L. casei* in Different Media.*

| Strain No. | $\mu\text{eq}^\dagger/\text{ml}$ | | |
|------------|----------------------------------|----------|----------|
| | Medium 1 | Medium 3 | Medium 4 |
| 697- 7 | 8.3 | 5.4 | 7.0 |
| 16 | 7.8 | 5.4 | 5.6 |
| 32 | 7.6 | 5.1 | 5.6 |

* Media 1 and 3 were the same as those described in Table I. Medium 4 contained 10 m μ moles of linoleic acid/ml but was otherwise the same as Medium 3.

† Growth promoting activity for strain 280-16A equivalent to that per μeq of D-lactic acid.

but also a considerable reversal of this effect in one of the strains (strain 697-7) by linoleic acid.

Discussion. It is clear from the experimental results that potassium acetate inhibition and its reversal by various fatty acids as described previously (1) is not characteristic of any single variety of *L. casei*, but rather is characteristic of a somewhat narrowly defined natural mixture of *L. casei* varieties. When the ratio of sensitive cells to resistant cells in the mixed inoculum is less than approximately 8:1 (Fig. 2), little or no inhibition due to potassium acetate is apparent. Mixed inocula with higher ratios of sensitive to resistant cells are markedly inhibited by potassium acetate, and reversal of the inhibition by either fatty acids or lithium and sodium ions (1) is evidently dependent on complex interactions of the different *L. casei* varieties.

It may be concluded from the present data that α -hydroxy acid-dependent varieties of *L. casei* occur naturally and differ only in minor respects, if at all, from strain 280-16 (4), which has previously been supposed to have resulted from an ultraviolet irradiation-induced mutation. The relatively high proportion of dependent varieties characteristic of our stock culture of *L. casei* is not, however, characteristic of 2 other stock cultures of *L. casei* which we have investigated recently in this regard. One of the latter, obtained through the courtesy of Dr. S. Shankman, ‡ originated from our stock in 1946, but has subsequently been carried in Yeast Dextrose Agar modified by the addition

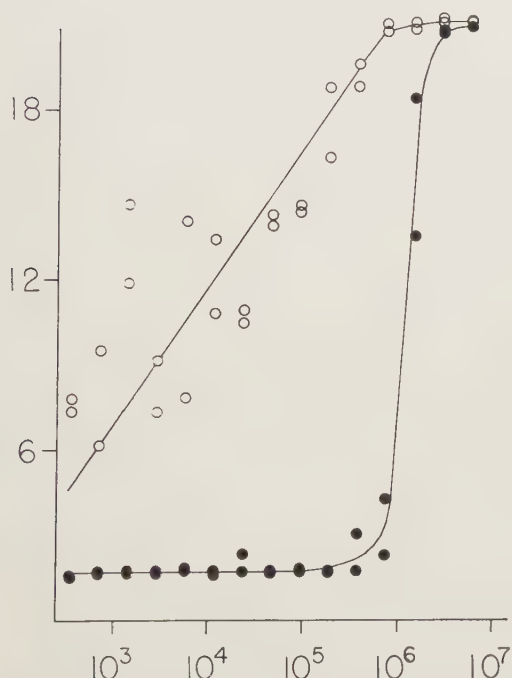


FIG. 2. Same as Fig. 1 with the exception that Medium 3 (Table I) was used in place of Medium 2.

‡ Shankman Laboratories, Los Angeles.

of 0.1% sodium acetate. The other was obtained as a lyophilized preparation (catalog number 7469) from the American Type Culture Collection. No dependent varieties were found among 60 isolates from each of these cultures. The question of whether our stock of *L. casei* contained a high proportion of dependent varieties in 1946 (when the Shankman stock originated from it) is apparently unanswerable, but it seems likely that this was true of our stock in 1952 on the basis of published data(4). It was reported(4) that 15 hour growth (but not later growth) of the "parent culture" was markedly stimulated by lactic acid, whereas we now know (unpublished data) that even the early growth of purified independent strains is not stimulated by α -hydroxy acids, and it may be inferred from this difference that the "parent culture"(4) contained a considerable proportion of dependent forms.

Summary. The stock culture of *L. casei* 7469 carried in this laboratory contained 6 to

45% (in different experiments) of forms resistant to inhibition by potassium acetate, yet inocula containing up to 12% of the resistant forms were markedly inhibited by potassium acetate apparently because of complex interactions between the resistant and sensitive varieties. Sensitive strains were found to be α -hydroxy acid-dependent (formerly this characteristic has been ascribed only to "mutant" strains). Sensitive strains, although predominating in the authors' stock culture, were not found among 60 isolates from either of two outside cultures of *L. casei* 7469.

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Renal Response to Non-Shocking Hemorrhage. Sodium Retention at Constant Perfusion Pressure.* (23762)

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(Introduced by Philip K. Bondy)

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Non-shocking hemorrhage (20 cc/kg for 35 minutes) decreases renal excretion of sodium despite autonomic blockade or renal denervation(1). Excretion of sodium increases again during retransfusion of the shed blood even when this is accomplished at a constant renal arterial blood pressure by inducing extrarenal vasodilatation with hexamethonium. The present experiments were carried out in order to study renal excretion of sodium during hemorrhage at constant arterial pressure, un-

der conditions where direct effects on the renal tubules of pharmacologic agents, or of the shed blood itself, were avoided.

Methods. Acute experiments were performed in dogs weighing from 10 to 20 kg anesthetized with 60 mg/kg of Dial intravenously. Inulin and para-aminohippurate in a solution of isotonic saline were administered via one femoral vein by means of a constant infusion pump. Renal arterial pressure was continuously recorded and monitored via a catheter inserted to the level of the renal vessels through the femoral artery. In some experiments the brachial artery pressure was recorded as an index of changes of the superior aortic blood pressure. A Sanborn Twin-Viso recorder and amplifiers were used with a Hathaway pressure capsule. Urine was col-

* Supported in part by grants from the Am. Heart Assn. and Nat. Heart Inst.

[†] During tenure of Established Investigatorship of Am. Heart Assn.

[‡] During tenure of Traineeship of Nat. Heart Inst.

[§] During tenure of Research Fellowship of Am. Heart Assn.

TABLE I. Changes in Functions of Innervated and Denervated Kidney Induced by Hemorrhage during Aortic Obstruction.

| % changes from control to test periods† | | | | | | | | | | | | | |
|---|--------|-------------|------|--------|-----|------|--------|-----|------|--------|-----|------|--------|
| Exp. groups and procedures* | Kidney | No. of obs. | ENa | | P‡ | EK | | P | FNa | | P | Cpah | |
| | | | Mean | ± S.D. | | Mean | ± S.D. | | Mean | ± S.D. | | Mean | ± S.D. |
| (A) Controls | | | | | | | | | | | | | |
| (1) (O-O) | Inn.§ | 20 | + 1 | ± 40 | | + 6 | ± 23 | | + 1 | ± 78 | | - 4 | ± 20 |
| | Den.§ | 9 | - 5 | ± 29 | | + 5 | ± 12 | | - 1 | ± 18 | | - 8 | ± 21 |
| (2) (O-BL) | Inn.§ | 14 | - 41 | ± 37 | .01 | - 9 | ± 63 | .40 | + 2 | ± 33 | .90 | - 10 | ± 17 |
| | Inn. | 4 | - 36 | ± 22 | " | - 5 | ± 10 | .10 | - 13 | ± 13 | .40 | + 23 | ± 37 |
| | Den.§ | 10 | - 48 | ± 25 | " | - 7 | ± 20 | .20 | - 4 | ± 16 | .70 | - 15 | ± 14 |
| (3) (O-CL) | Inn. | 11 | + 34 | ± 68 | .10 | + 35 | ± 62 | .10 | + 4 | ± 16 | .80 | - 1 | ± 25 |
| | Den. | 4 | + 25 | ± 77 | .40 | - 15 | ± 46 | .40 | - 16 | ± 23 | .20 | - 11 | ± 22 |
| (B) Exps. with hemorrhage at constant renal arterial pressure | | | | | | | | | | | | | |
| (4) (O-BL/CL) | Inn. | 7 | - 41 | ± 23 | .01 | - 10 | ± 22 | .10 | - 13 | ± 14 | .40 | + 8 | ± 26 |
| | Den. | 4 | - 33 | ± 28 | " | - 23 | ± 19 | .02 | - 20 | ± 24 | .10 | - 14 | ± 18 |
| (5) (O-BL/BaL) | Inn. | 19 | - 24 | ± 22 | .03 | 0 | ± 41 | .90 | - 82 | ± 23 | .01 | - 10 | ± 27 |
| | Den. | 9 | - 59 | ± 19 | .01 | - 18 | ± 29 | .02 | - 18 | ± 13 | .10 | - 26 | ± 25 |
| (BL/BaL-O) | Inn. | 17 | + 90 | ± 51 | .01 | + 20 | ± 54 | .40 | + 16 | ± 53 | .50 | + 21 | ± 68 |
| | Den. | 9 | + 18 | ± 50 | .20 | 0 | ± 20 | " | 0 | ± 24 | .90 | + 8 | ± 25 |

* Letters and numbers designate exp. groups as described under *Methods*. Symbols designating procedures used are: O-O = Spontaneous changes; no test procedure. O-BL = Changes with hemorrhage. O-CL = Changes with aortic clamping. O-BL/CL = Changes with hemorrhage while aorta clamped. O-BL/BaL = Changes with hemorrhage while aortic balloon deflated. BL/BaL-O = Changes when shed blood retransfused while aortic balloon inflated.

† ENa = Excretion rate, sodium. EK = Excretion rate, potassium. FNa = Filtration rate, sodium. Cpah = Para-aminohippurate, clearance.

‡ P = Probability of chance diff. between test and control groups.

§ Control data derived from previous report(1). || Control data from present experiments.

lected from each kidney by ureteral cannulation, each period of collection lasting 15 to 30 minutes. Aqueous heparin (0.2 mg/kg intravenously) was used as an anticoagulant. Bleeding, when desired, was performed by allowing blood to flow via a polyethylene cannula inserted into the brachial or femoral artery into an inverted capped silicone-coated bottle containing air at a pressure adjustable through a side arm connected to a manometer and pressure bulb. Bleeding volumes, (at 1.5 to 3% of the body weight), were sufficient to cause renal retention of sodium on the basis of past experience(1). Renal arterial pressure was maintained constant by appropriate adjustment of one of two types of mechanical aortic obstruction: 1. Compression of aorta by clamp applied just below renal arteries (O-BL/CL, Table I). Only small hemorrhages (< 2% of body weight) were possible by this procedure without a fall in renal arterial blood pressure. 2. Graded inflation of rubber balloon at end of catheter inserted via the left carotid artery to level of the diaphragm. Prior to bleeding, this balloon was

inflated sufficiently to lower renal arterial pressure to about 90 to 100 mm Hg (mean), this level being controlled by constant monitoring with a direct-writing pressure recorder (see above). After at least one-half hour at this reduced arterial pressure, several control periods were obtained, following which bleeding was allowed up to 3% of the body weight, while the aortic balloon was gradually deflated so as to maintain renal arterial pressure constant ((O-BL/BaL), Table I). After several periods, the shed blood was retransfused while the balloon was appropriately re-inflated ((BL/BaL-O), Table I). Large changes of blood volume were thereby possible without changing renal arterial perfusion pressure (and without using autonomic blocking agents, as in prior studies). In experiments designated by "Den", Table I, preliminary unilateral left renal denervation was carried out one to two weeks beforehand in 19 dogs by complete separation of each kidney from its attachments, except for the artery, veins and ureter, 1 to 2 cm of which were stripped of all visible nerve fibers and painted

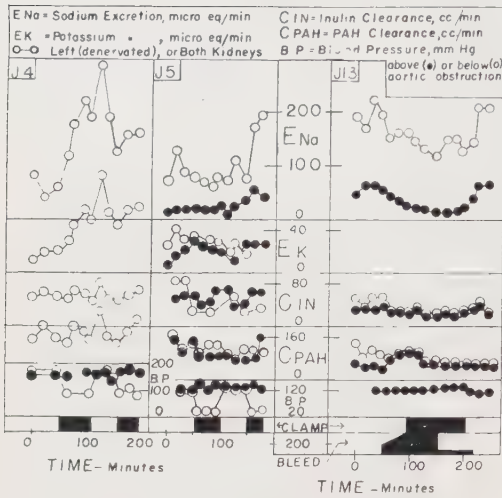


FIG. 1. Changes in functions of innervated and denervated kidneys induced by hemorrhage during aortic clamping, and by aortic clamping alone.

with absolute alcohol. The efficacy of this procedure has been previously verified. Clinical analyses and calculations were the same as those described in a previous report from this laboratory.

Results. *A. Control Experiments* (Summarized in Table I). Hemorrhage ((O - BL), Table I) of the degree used throughout our study (1.5 to 3% of body weight, representing about 20 to 35% of total blood volume) reduced excretion of sodium in 4 new experiments as had been observed previously (1). Clamping of the aorta just below the renal arteries ((O - CL), Table I) caused no significant change in sodium excretion or in other renal functions (Fig. 1) and only small, transient increases of superior aortic (brachial artery) blood pressure. Inflation of the aortic balloon until the renal arterial pressure was reduced to about 100 mm Hg decreased the excretion rate of sodium by both the denervated and the innervated kidney. The steady values of sodium excretion obtained after one-half hour at reduced renal arterial pressure were used as controls for the subsequent experimental periods.

B. Experiments with hemorrhage at constant renal arterial pressure (Table I). Hemorrhage at constant renal arterial pressure maintained either by aortic clamping ((O - BL/CL), Table I), or by deflation of an aor-

tic balloon ((O - BL/BaL), Table I) reduced excretion of sodium by both the innervated and the denervated kidney (Fig. 1 and 2). Excretion of potassium by the denervated kidney (but not by the innervated kidney) was also reduced. Renal blood flow was not significantly changed. Glomerular filtration of sodium was reduced significantly only in the innervated kidney when renal arterial pressure was controlled by an aortic balloon ((O - BL/BaL), Table I). Reflex changes of (? predominantly efferent) arteriolar tone may have changed glomerular filtration in these experiments, in response to the larger hemorrhages possible when an aortic balloon, rather than an aortic clamp, was used to control renal arterial pressure.

Retransfusion of the shed blood at constant renal arterial pressure, maintained by re-inflation of the aortic balloon ((BL/BaL - O), Table I) increased sodium excretion in the innervated kidney (Fig. 2). Significant changes of other functions were absent. In several experiments, sodium excretion also increased in the denervated kidney (Fig. 2), but the average change of all experiments was not significant (Table I).

Discussion. The experiments of this report indicate that the excretion of sodium by the innervated or by the denervated kidney declines during hemorrhage, even though the

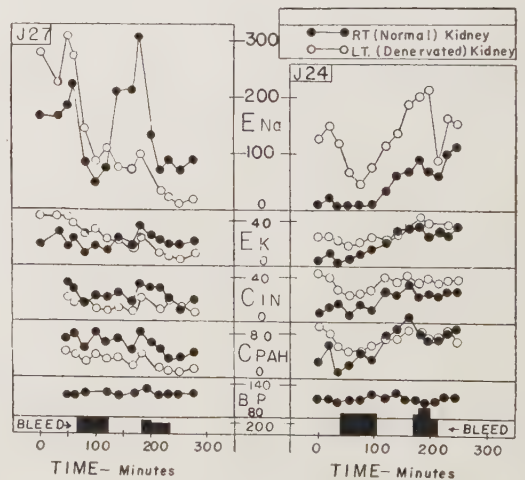


FIG. 2. Changes in function of innervated and denervated kidneys induced by hemorrhage during controlled deflation of an aortic balloon. (Symbols as of Fig. 1.)

renal arterial pressure is maintained at a constant value by mechanical means. These observations might be explained on the basis of an extrarenal receptor located in the upper aortic distribution, sensitive to diminished blood flow or volume, and regulating renal tubular reabsorption of sodium via humoral or endocrine secretion. Recent experiments have, in fact, disclosed that an increased secretion of aldosterone and hydrocortisone is induced by hemorrhage either directly or via ACTH(2,3). However, the renal retention of sodium elicited by hemorrhage in the present experiments was more rapid than might have been expected as a result of the secretion of presently known adrenal steroids. Furthermore, no changes of potassium excretion characteristic of adrenal steroid action(4) were observed (Table I). Consequently, the present experiments reinforce the previous proposal(1) that hemorrhage may cause an intrarenal redistribution of blood flow, perhaps toward vessels of less than average resistance perfusing tubules with more than average sodium reabsorbing activity. Current studies of the renal hematocrit during isobaric hemorrhage lend further support to this concept.

Summary. The renal excretion of sodium and other renal functions were studied in dogs during non-shocking hemorrhage, while renal arterial pressure was maintained constant by controlled partial mechanical obstruction of the aorta. In some experiments, one kidney was denervated beforehand. The excretion of sodium declined in the absence of measurable changes in glomerular filtration or renal plasma flow. The explanation is proposed that hemorrhage causes an intrarenal redistribution of blood flow which favors tubules with more than average sodium reabsorbing activity, although steroid effects on tubular function cannot be excluded.

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Yolk-sac Perfusion of Chick Embryos: Effects of Various Media on Survival.* (23763)

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The difficulty of maintaining the delicate relationships of the chick embryo to its membranes and to its nutrient supply have until recently precluded direct studies of embryo nutrition in the intact egg. Important data (1) have been obtained indirectly by feeding the laying hen diets deficient in vitamins and minerals, and determining the subsequent effect on development of the embryo. This method cannot be used to study the embryo's supply of amino acids and most energy

sources, however, because deficiencies of these nutrients in the hen's diet result in reduced egg production, not in the production of eggs containing lower concentrations of nutrients. One direct approach has been the *in vitro* cultivation technic, which Spratt(2) has used to study the nutrition of chick embryos during early development (18 to 58 hours of incubation). New(3) has recently developed a method of explantation which allows development to proceed for 60 hours, 40 hours of which are *in vitro*. The objectives of the methods developed in this laboratory have been to remove the embryo's nutrients, and

* This work was supported by grant from Nutrition Fn.

replace them with a medium of known composition, without disturbing the normal relationship of the embryo to the intact egg. In order to do this, an electrosurgical unit was used to form a channel of coagulated albumen leading through the shell membranes, the albumen, the vitelline membrane and finally into the yolk. The yolk could then be flushed out through the channel and replaced by other yolk or by a replacement medium. When donor yolk was supplied to 3-day chick embryos from which at least 95% of the original yolk had been removed, normal development occurred, including the hatching process(4). After developing the basic technic of yolk replacement we conducted a series of experiments using various media as yolk-replacing materials. Prior to the experiments reported here, however, we had not demonstrated distinct differences among media (except where tolerances for amino acids or heavy metals were exceeded and rapid embryo death ensued). Most of these early trials were performed with defined media similar to that used in the experiments reported in this paper. In this work, the yolk sac was flushed for a few minutes with 15 to 25 ml of medium, after which the hole was closed with plastic adhesive tape. Some embryos withstood 9 flushes of this sort, at 6 hour intervals, before they died. The survival time was the same whether the medium was complete or was deficient in lysine, or whether Earle's balanced salt solution(5) alone was used. We next resorted to continuous yolk-sac perfusion whereby we compared various defined media to balanced salt solutions, but again no differences in survival were observed. Finally, dilute yolk was used in the replacement medium as we report in this paper.

Methods. The basic technics of coagulation and yolk removal have already been described in detail(4). Certain modifications were made to permit the continuous passage of fluids through the yolk sac. In the present study, most of the yolk was removed after 3 days of incubation, but perfusion was not started until the embryos were 4 days old. This schedule permitted the embryo to recover from the operation, facilitated the selection of more uniformly vigorous embryos,

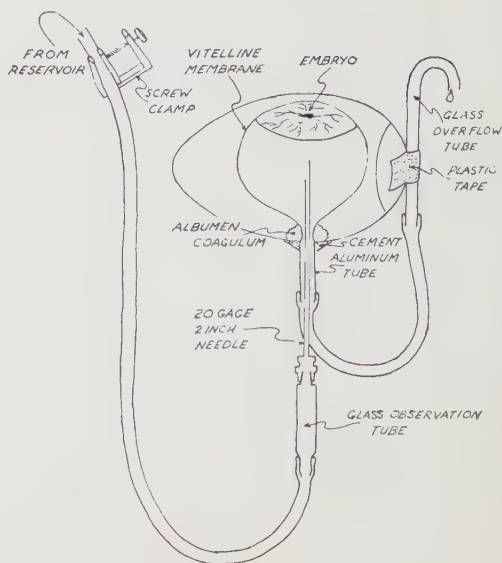


FIG. 1. Yolk-sac perfusion preparation.

and provided time for the residual yolk to accumulate near the opening of the coagulum, with the result that most of it was removed as soon as flushing with the medium was started. After connection between yolk and shell was established(4), an aluminum tube, 4 mm x 15 mm, beveled at both ends, was inserted approximately 4 mm into the hole and was fastened firmly in place with model-airplane cement. Most of the yolk was then flushed out as before, using Earle's balanced salt solution(5). A rubber tube, attached at one end to a glass overflow tube, was slipped over the exposed end of the aluminum tube and taped onto the shell (Fig. 1). These operations were carried out at room temperature, but as soon as the overflow tubes had been attached, the eggs were placed in a room maintained at 100°F and remained there for duration of experiment. The perfusion apparatus consisted of reservoir attached by rubber tubing to stainless steel manifold pipe fitted with small aluminum tubes. Rubber tubing connected these small tubes to glass observation tubes and to 2 in., 20 gauge hypodermic needles. The needles were inserted through rubber overflow tubes and into center of each yolk sac, as shown in Fig. 1. A screw clamp was used to maintain flow rate to each individual egg. **Materials.** Yolk used as part of the perfusion medium was har-

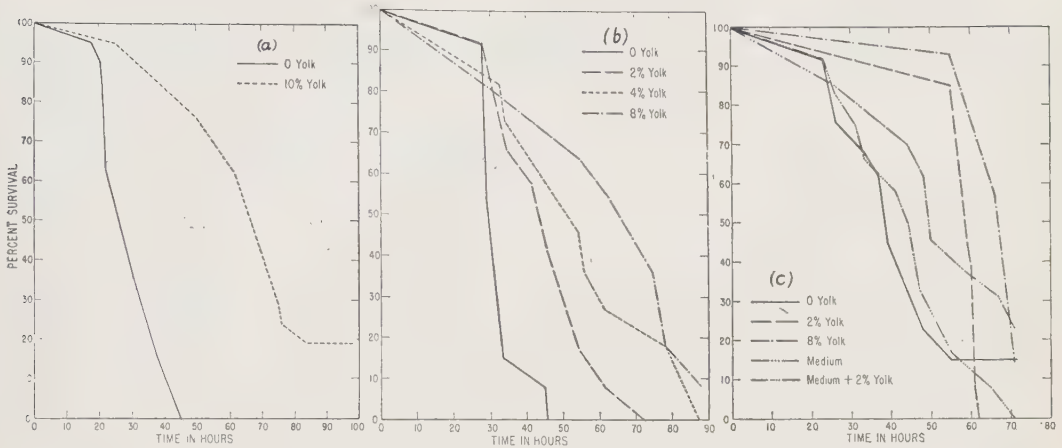


FIG. 2. Effect of varying the perfusion medium on survival of 4-day chick embryos. (a) There were 19 embryos on 0 yolk and 21 on 10% yolk. Perfusion schedule was for alternating 8-hr periods at the rate of 0.5 ml/min. (b) There were 11-13 embryos/group. Same schedule as (a). (c) There were 12-14 embryos/group. Perfusion schedule was for alternating 4-hr periods.

vested aseptically from fresh eggs and was added to Earle's salt solution or to the synthetic medium, which had been sterilized by filtration. The defined medium, which was similar to that of Evans *et al.* (6), contained the following materials, expressed as mg per 100 ml. (The amino acids were all of the L-configuration.): Alanine, 3.38; arginine hydrochloride, 6.30; asparagine, 1.20; aspartic acid, .30; cystine, 2.25; glutamic acid, 3.00; glycine, 8.10; histidine, 3.60; isoleucine, 4.50; leucine, 6.75; lysine hydrochloride, 6.30; methionine, 2.70; phenylalanine, 4.50; proline, 6.00; serine, 3.75; threonine, 3.75; tryptophan, 2.70; tyrosine, 4.50; valine, 4.50; thiamin hydrochloride, .0050; riboflavin, .0050; pyridoxine hydrochloride, .0125; pyridoxal hydrochloride, .0125; niacin, .0125; niacinamide, .0125; calcium pantothenate, .0050; biotin, .0050; folic acid, .0050; choline chloride, .250; i-inositol, .0250; p-aminobenzoic acid, .0250; vitamin A alcohol, .0500; Vit. D-3, .0500; menadione, .0050; d-alpha-tocopheryl acetate, .0050; methyl linoleate, .60; cholesterol, .40; Tween 80, 4.50. Earle's salt solution (5) at normal concentration was used in the defined medium as well as in the dilute yolk media. The media and flushing solution contained 66 mg of streptomycin and 9.5 mg of erythromycin per 100 ml. After each 8 hours of perfusion, the media were replaced with fresh mixtures.

Results. The data presented in Fig. 2a indicate that embryos survived for longer periods when perfused with 10% yolk in Earle's salt solution than when Earle's salt solution alone was used. Among the embryos perfused with the medium containing no yolk, death was first observed 18 hours after the start of perfusion, and all had died by 45 hours (141 hours total incubation time). None of the embryos receiving 10% yolk died before 30 hours and some were still alive after 96 hours (192 hours total incubation time), when the experiment was terminated. In this study, the yolk sacs were perfused at a rate of 0.5 ml/min. for 8 hours, then were left undisturbed for an alternate 8-hour period throughout the 96 hours of the experiment. Time of death was estimated by candling appearance, with particular reference to the appearance of the blood vessels.

The second experiment (Fig. 2b) showed the effect of three levels of yolk and of Earle's salt solution alone on survival. The procedures were the same as those of the first experiment. None of the embryos that received no yolk survived beyond 45 hours after the start of the experiment; some of those that received 2% yolk and 4% yolk survived as long as 73 and 87 hours respectively. One of the embryos on the 8% yolk was still alive at 87 hours, when the experiment was terminated.

Fig. 2c shows the results of a study comparing levels of yolk (in Earle's salt solution) and the defined medium. The principal difference from the preceding experiment was that the embryos were perfused for alternating 4-hour periods instead of for 8-hour periods. The embryos receiving no yolk again showed a relatively high death rate, but two of these 13 embryos were alive at the end of the experimental period (71 hours). The defined medium plus 2% yolk allowed poorer survival than the yolk-free defined medium, while the 2% yolk in Earle's salt solution was much better than either of the 2 media containing the known nutrients. The embryos that received 8% yolk survived longer than the embryos on other media.

In all of these studies, the embryos developed normally until death intervened.

Discussion. The results of the second experiment, in which various levels of yolk were compared (Fig. 2b), agree with the data obtained in the first experiment (Fig. 2a). In the third experiment (Fig. 2c) the embryos given 2% yolk or no yolk survived longer than similar embryos in the second experiment. We attribute these apparent discrepancies to incomplete flushing, because at the end of the experiment the yolk sacs of several embryos that had survived on low levels of yolk or on the defined medium contained relatively large amounts of the original yolk.

A comparison of Fig. 2b and 2c, at the 2% and 8% yolk levels, suggests a relationship between the schedule of perfusion periods and yolk concentration. Alternating 8-hour periods (Fig. 2b) may have resulted in more rapid depletion of 2% yolk than of 8% yolk during the 8-hour, non-perfusion period. When perfusion was conducted for alternating 4-hour periods, however, the response may have reflected differences in yolk concentration rather than depletion effects. This would explain the relatively long survival of the 2% yolk embryos in the last experiment.

The results obtained in the present study

show that it is technically possible to influence embryo survival by varying the yolk-sac perfusion medium. The defined medium used here, in contrast to very dilute yolk, was clearly not a satisfactory yolk substitute. Its addition to the dilute yolk markedly decreased the value of the dilute yolk medium, thus indicating some imbalance of nutrients, or possible toxicity.

Summary. 1) Yolk-sac perfusion, a new technic which permits the continuous or intermittent passage of fluids through the yolk sac of 4-day chick embryos, has been used to study survival of chick embryos perfused with varying concentrations of yolk. 2) When a yolk-free, balanced salt solution was perfused for 8 hours at 0.5 ml/min., alternating with 8 hours of non-perfusion, almost all embryos died between 20 and 45 hours. Survival time increased in proportion to yolk concentration up to 8% yolk, at which level some embryos were alive 96 hours after perfusion was started. 3) The defined medium was not a satisfactory yolk substitute. The combination of medium and dilute yolk resulted in even poorer survival than that obtained with yolk alone, thus indicating an imbalance or toxicity of one or more components of the medium.

The excellent technical assistance of T. L. Lau, B. D. Lundholm, and H. I. Fritz is gratefully acknowledged. Streptomycin was kindly provided by Merck and Co.; erythromycin by Abbott Laboratories.

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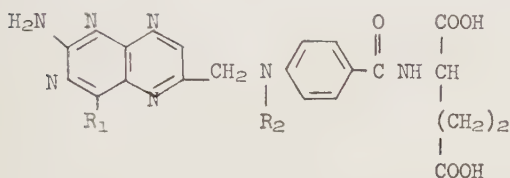
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Inhibition of Dihydrofolic Reductase by Aminopterin and Amethopterin.*†

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(Introduced by C. A. Finch)

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Since the folic acid antagonists, Aminopterin (I, $R_1 = \text{NH}_2$, $R_2 = \text{H}$) and Amethopterin (I, $R_1 = \text{NH}_2$, $R_2 = \text{CH}_3$) have proved to be of considerable value in the treatment



of certain human and experimental leukemias (see(1) for a general review of this subject), it would be of interest to ascertain the specific site of action of these antagonists at the enzymatic level. Extensive studies by Nichols (2) have shown that the inhibition occurs at some point in the conversion of the vitamin, folic acid, (I, $R_1 = \text{OH}$, $R_2 = \text{H}$) to folinic acid (N^5 -formyl, 5,6,7,8-tetrahydrofolic acid). From recent findings, reviewed elsewhere(3,4, 5), it is now known that 5,6,7,8-tetrahydrofolic acid is the coenzyme form of folic acid (*i.e.* the carrier for one-carbon fragments at the oxidation level of formate and formaldehyde), and that folinic acid is probably a stable storage form for tetrahydrofolic acid. These relationships are outlined in equation (1):



As a secondary effect, understandable from the above considerations, inhibition by folic acid antagonists has been observed with certain of the metabolic reactions which involve

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† Paper VI in *Folic Acid Coenzymes and Active One-Carbon Units*; for Paper V see M. J. Osborn, E. N. Vercamer, P. T. Talbert, and F. M. Huennekens, *J. Am. Chem. Soc.*, 1957, v79, 6565.

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a folic acid coenzyme, *e.g.* incorporation of formate into purines(6) or thymine(7), or interconversion of serine and glycine(8). The second step in the conversion of folic acid to the coenzyme form (*i.e.* reduction of FH_2 to FH_4)§ is mediated by a TPN-linked dehydrogenase, which has recently been obtained in purified form from chicken liver(9).†† The stoichiometry of the reaction is shown in equation (2):



In this communication evidence is presented that the dihydrofolic reductase is inhibited non-competitively by extremely low concentrations of Aminopterin and Amethopterin.

Materials and methods. TPNH was a product of the Sigma Chemical Co. Folic Acid was obtained from the California Foundation for Biochemical Research, 9, 10-dimethyl folic acid from the Lederle Laboratories, Aminopterin from the Mann Research Laboratories, and Amethopterin from the Bios Laboratories. The absorption spectra of Aminopterin and Amethopterin in 0.1 N NaOH or 0.1 N HCl agreed well with the published spectra(10) of these compounds. Paper chromatographic examination of the antagonists in 3 solvent systems ((a) 1 M sodium formate containing 2% formic acid; (b) 0.1 M glycine, pH 9.5, containing 2% sodium versenate; and (c) ethanol:water (70:30 v/v)) revealed in each case a principal component with traces of other fluorescent materials; such inhomoge-

§ The following abbreviations are used: F, folic acid; FH_2 , 7,8-dihydrofolic acid; FH_4 , 5,6,7,8-tetrahydrofolic acid; f^5FH_4 , f^{10}FH_4 , N^5 -formyl and N^{10} -formyl FH_4 ; ATP, ADP, adenosine tri- and diphosphate; P_i , inorganic phosphate; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide.

†† S. Futterman (*J. Biol. Chem.*, 1957, v228, 1031) has also studied the enzymatic reduction of folic acid in chicken liver preparations and has observed a similar inhibition by the folic acid antagonist, Aminopterin.

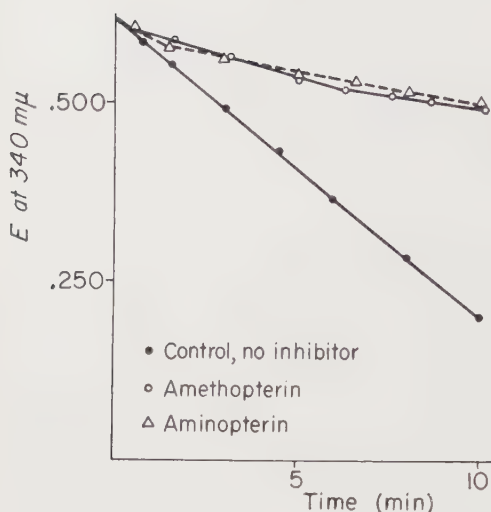


FIG. 1. Standard spectrophotometric assay system contained (in 1 cm Corex cuvette) .03 ml of purified FH_2 reductase, .07 μmoles of FH_2 , .10 μmoles of TPNH, 50 μmoles of phosphate buffer, pH 7.5, 10 μmoles of 2-mercaptoethanol, and 1.4×10^{-4} μmoles of Aminopterin or Amethopterin, where indicated, in volume 1.25 ml. Decrease in light absorption (E) at 340 $m\mu$ was measured against time, and values obtained were corrected for small changes in separate, blank cuvettes omitting FH_2 or TPNH.

neity is routinely observed during paper chromatography of folic acid compounds, and is reminiscent of the known lability of the flavin nucleotides(11) under similar conditions. FH_2 was prepared by catalytic hydrogenation of folic acid in 0.1 N NaOH, essentially according to the method of O'Dell *et al.*(12).|| The purification of the FH_2 reductase, and the spectrophotometric assay for reaction(2) are described elsewhere(9).

Results. In the presence of purified reductase, FH_2 is rapidly reduced by TPNH, as shown in the control curve of Fig. 1. The addition of Aminopterin or Amethopterin, each at a final concentration of 1.1×10^{-7} M, to the assay system, results in an inhibition of approximately 70%. Higher concentrations (*ca.* 5×10^{-7} M) of the inhibitors produce complete inhibition. It should be noted that the level of substrate, FH_2 , used in the experiment (5.3×10^{-4} M) is far above that necessary to saturate the enzyme ($K_m = 5.0 \times 10^{-7}$ M, see below).

|| We are indebted to Dr. P. T. Talbert and Mr. J. G. Ozols for carrying out this preparation.

A more detailed kinetic study of inhibition by Aminopterin and Amethopterin is presented as the conventional Lineweaver-Burk double reciprocal plots in Fig. 2. The affinity of the enzyme for both inhibitors is extraordinarily high. Calculation of the inhibitor constants yields K_I values of 1.0×10^{-9} M and 2.3×10^{-9} M for Aminopterin and Amethopterin, respectively. It is of interest that the Michaelis constant for FH_2 , $K_m = 5.0 \times 10^{-7}$ M,|| is also unusually low for a substrate of a pyridino-protein enzyme. The fact that the inhibitor curves do not pass through a common intercept on the ordinate axis suggests that both inhibitors act *non-competitively*, rather than competitively. Although folic acid and 9,10-dimethyl folic acid have been shown also to inhibit the dihydrofolic reductase(9), they are effective only at concentrations greater than 10^{-6} M. Thus, the

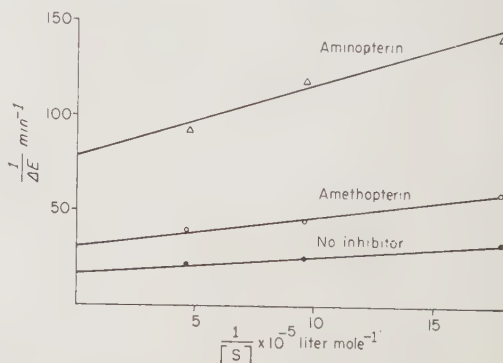


FIG. 2. Silica cells with 10 cm light path initially contained 0.1 ml of purified FH_2 reductase, 0.13 μmoles of TPNH, and 9×10^{-5} μmoles of Aminopterin or 4.4×10^{-5} μmoles of Amethopterin, where indicated, in 25 ml of 0.05 M phosphate-0.01 M 2-mercaptoethanol, pH 7.5. The reaction was started by addition of FH_2 to final conc. of 5.6×10^{-7} M, 1×10^{-6} M, or 2.2×10^{-6} M. Decrease in light absorption at 340 $m\mu$ (ΔE) was measured against time, and initial velocity (expressed as $\Delta E/\text{min.}$) was corrected for the small blank oxidation of TPNH in the absence of FH_2 . In the figure the reciprocals of velocity and substrate conc. $[S]$ are plotted as ordinate and abscissa respectively. K_m values for FH_2 , and K_I values for inhibitors were calculated by the conventional Lineweaver-Burk method. The extremely low values for K_m and K_I made it necessary to work at high dilutions of FH_2 . This, in turn, produced only small changes in TPNH (*cf.* the stoichiometry of equation (2)) and necessitated the use of long optical paths (10 cm) to achieve measurable values of ΔE .

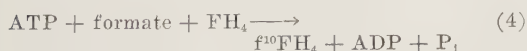
|| Separate determinations of K_m for FH_2 have yielded values of 6.0×10^{-7} M. and 3.5×10^{-7} M.

degree of inhibition observed with Aminopterin and Amethopterin is several orders of magnitude greater than could be accounted for on the basis of any possible contamination by folic or methyl folic acids. Moreover, the possibility that other trace contaminants in the antagonists are the actual causative agents for the observed inhibition is unlikely since the inhibitor constants would then be even lower, *i.e.* 10^{-10} or 10^{-11} M.

The inhibition of the dihydrofolic reductase is the first example of an appreciable effect by low levels of Aminopterin and Amethopterin on an isolated enzyme system. Although several investigators(2) have observed an inhibition by these folic acid antagonists on the conversion of folic acid to folinic acid, the exact localization of the block has required the characterization of the individual enzymes participating in the multi-step process shown in equation (1). The reduction of folic acid to FH_2 (*cf.* equation (3)) has recently been studied by Wright and Anderson(13) in extracts of *E. coli*.



It was reported that this reaction, which requires some component of the pyruvic oxidase system to supply the reductive power, is not inhibited by Aminopterin. The formate-activating enzyme(14), which mediates the formation of N^{10} -formyl FH_4 according to equation (4) is also not sensitive to



Aminopterin or Amethopterin.** The conversion of f^{10}FH_4 to f^5FH_4 (folinic acid) is still not well understood.

Therefore, it appears that the primary site of action of the 4-amino analogues of folic acid in blocking the conversion of folic acid to its coenzymatically active forms is probably only at the FH_2 reductase reaction. Fur-

thermore, the finding that the inhibition is *non-competitive* with the K_i values of approximately 10^{-9} M adequately accounts for both the physiological potency of the drugs, and also for the relative ineffectiveness of folic acid, even in massive doses, in reversing the toxicity of the antagonists.

Summary. The enzyme, dihydrofolic reductase, which catalyzes the TPNH-linked reduction of dihydrofolic acid to tetrahydrofolic acid, is inhibited non-competitively by the folic acid antagonists, Aminopterin and Amethopterin. Values of 1×10^{-9} M and 2.3×10^{-9} M were obtained for the inhibitor constants (K_i) of Aminopterin and Amethopterin, respectively.

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** Unpublished experiments with Dr. H. R. Whiteley.

Uptake of Radiosulphur in Growing Bones of Cockerels Treated with Cortisone and 17-Ethyl-19-Nortestosterone.* (23765)

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It is apparent that cortisone exhibits catabolic effects both in birds and in mammals. Recently Hublé has shown that chondrogenesis in proliferation zones of proximal and distal ends of the 3 long bones of the cockerel's leg was inhibited by cortisone treatment (1). This author was using as criteria of hormonal effect, the histological changes induced in cartilage and bone. We were able to demonstrate that catabolic and anabolic changes in cartilage and bone of rat, may be studied satisfactorily with S^{35} labelled sulphate uptake procedure (2-6). It is well established that the selective deposition of radiosulphur in certain tissues is due to utilization of sulphate ions in the synthesis of chondroitin sulphate of connective tissue ground substance and in collagen (7,8). We showed that S^{35} uptake by the healing fractured bone in rats may be significantly inhibited by cortisone (4,5) and stimulated by certain anabolic steroids (2,3). This was interpreted that cortisone inhibited and anabolic steroids stimulated production of certain mucopolysaccharides which are essential in collagen fibrillogenesis and which specifically incorporate labelled sulphate. The same procedure was also used to investigate the catabolic effect of AT-10 (dihydrotachysterol in rats) (6). It was shown that an anabolic steroid 17-ethyl-19-nortestosterone (Nilevar) (9) counteracted the catabolic effects of both cortisone (4,5) and AT-10 (6). It was apparent from these results that the hormonal effect on bone repair may be studied with radiosulphur, and that this procedure may be also practical for the study of chondrogenesis and osteogenesis.

The purpose of the present experiment was to compare effects of cortisone and 17-ethyl-19-nortestosterone on the S^{35} uptake by the 3 long bones of the young cockerel's leg. An

attempt was also made to offset the catabolic effect of cortisone by simultaneous treatment of birds with 17-ethyl-19-nortestosterone.

Materials and methods. Cockerels of White Rock-Broiler strain, were obtained from a commercial hatchery when 3 days old and immediately confined to thermostatic brooders (Start-Gro) 24 x 36 inches capacity. The birds were kept in groups of 15 per brooder, and received routine chicken food with tap water *ad libitum*. Hormones were injected, beginning the sixth day of bird's life, and administered during the 3 following weeks, 4 doses the first week and 3 doses weekly for the next 2 weeks. Following 4 groups were studied: 1. Control group; no treatment. 2. Given cortisone acetate (Merck, Co.); 5 mg in 0.5 cc saline, subcutaneously. Total dosage 50 mg/bird. 3. Given 17-ethyl-19-nortestosterone (Nilevar, Searle & Co.); 5 mg in 0.2 cc of sesame oil, intramuscularly. Total dosage/bird 50 mg. 4. Given both cortisone and 17-ethyl-19-nortestosterone, as Groups 2 and 3. Isotope was administered 24 hours after last dosage of hormone. Radiosulphur (S^{35} in H_2SO_4 , Atomic Energy, Canada) was injected subcutaneously, one mc/kg of bird's body weight. The dose was dissolved in 5 ml of distilled water together with 40 mg of sodium sulphate (10). Immediately after sacrifice the femur, tibiotarsus and tarso-metatarsus were separated, cleaned from adhering tissues and dried at room temperature 24 hours, following which their weights were recorded. The bones were then dissolved by nitric-perchloric acid wet-digestion procedure described previously (10). The final precipitate of radioactive barium sulphate was transferred to planchettes and counting carried out using a thin mica window Tracerlab G-M tube. A Tracerlab Superscaler S.C. 18A was used for recording. Results were calculated in counts/minute (c.p.m.) per 100 mg of tissue. The changes

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in body weight were also recorded and presented as percentages of gain in body weight, during the experimental period of 3 weeks.

Results. Two tables summarize our results. The gain of body weight observed in 4 groups of birds during the 3-week experimental period is recorded in Table I. It may be noted that normal control birds gain about 265% of body weight as compared with about half of this value found in cortisone-treated cockerels. Anabolic steroid Nilevar not only promotes body growth but also partially alleviates the inhibitory effect of cortisone.

Table II records the data on measurement of radioactivity of bones. Because dosage of isotope corresponded to body weights of cockerels (1 mc/kg B.W.), the recording of results in c.p.m./100 g of bone may be considered as most appropriate. It is evident that cortisone significantly reduced radiosulphur uptake in bones, as compared with normal control. The effect of cortisone is more pronounced here than the effect of this catabolic hormone on body growth.

Anabolic steroid 17-ethyl-19-nortestosterone significantly promoted the binding of labelled sulphate by the studied bones. Simultaneous treatment with both steroids offset the effect of cortisone.

Discussion. The inhibitory effect of cortisone on chondrogenesis and osteogenesis in birds, demonstrated by histological technics (1), seems also to be proved in our experimental conditions. Radiosulphur uptake procedure is apparently an adequate method for study of hormonal effect on production of mucopolysaccharides in connective tissue (11). This method shows that cortisone inhibits and

TABLE II. Radiosulphur Uptake by 3 Long Bones (Femur, Tibiotarsus and Tarso-metatarsus) of Growing Cockerels. Expressed in cpm (count/min.) per 100 mg of bone.

| Treatment | No. of birds | cpm \times 100 |
|--|--------------|-----------------------------------|
| Control | 15 | 6.20 \pm .87 * (4.70-7.93) † |
| Cortisone, 50 mg | 15 | 1.98 \pm .26 (1.56-2.50) |
| 17-ethyl-19-nortestosterone, 50 mg | 12 | 15.87 \pm 3.2 (12.8-20.6) |
| Cortisone, 50 mg, and 17-ethyl-19-nortestosterone, 50 mg | 12 | 10.75 \pm 1.2 (8.9-12.0) |

* Mean \pm S.D.

† Range.

17-ethyl-19-nortestosterone (Nilevar) promotes synthesis of collagen during the process of growing of long bones in young cockerels. Combined therapy protects connective tissue of growing bones against the catabolic effect of cortisone. It is apparent that Nilevar has highly anabolic property not only during the process of healing of connective tissue (2,3) but may also accelerate its growth in young animals.

Summary. 1. The effects of cortisone and 17-ethyl-19-nortestosterone (Nilevar) on radiosulphur uptake by the three long bones of the young cockerel's leg, were investigated. Cortisone diminished and Nilevar increased the binding capacity of labelled sulphate by the growing bones of birds. This was interpreted as the catabolic and anabolic effect of these hormones respectively, on synthesis of certain mucopolysaccharides of growing connective tissue, which specifically incorporate radiosulphur. 2. The anabolic steroid 17-ethyl-19-nortestosterone counteracted the catabolic effect of cortisone on growing bone, when both hormones were administered simultaneously.

TABLE I. Effect of Various Steroids on Gain in Body Weight in Cockerels. Expressed as % of gain after 3 weeks of treatment.

| Treatment | No. of birds | % increase in body wt |
|--|--------------|-----------------------|
| Control (no treatment) | 15 | 265 \pm 10 * |
| Cortisone, 50 mg | 15 | 140 \pm 9 |
| 17-ethyl-19-nortestosterone, 50 mg | 12 | 300 \pm 12 |
| Cortisone, 50 mg, and 17-ethyl-19-nortestosterone, 50 mg | 12 | 188 \pm 11 |

* Mean \pm S.D.

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Nutrition Studies in the Cold. II. Curative Effect of Cold on Fat Fatty Livers.* (23766)

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The comparative response of rats maintained at 25° and at 1° to variations in protein and fat content of the diet has been described recently(1). Diets of a low protein or high fat content produced elevated levels of liver fat at 25° while at 1° the liver fat was within the normal range at all protein and fat levels which were employed. It has been demonstrated that in addition to the preventive effects of supplementary choline and methionine in hypolipotropic diets, these lipotropic factors have a curative effect on fat fatty livers, *i.e.*, will decrease an elevated level of liver fat due to previous ingestion of a hypolipotropic diet(3).

It became of interest to determine if cold also had a curative effect on fat fatty livers† as well as the preventive effect described recently(1). The data presented below clearly demonstrate that cold exhibits both of these characteristic effects of the other lipotropic factors.

Methods. Young male rats (50-80 g) of the Carworth strain were used. The care of the animals and the general experimental conditions were the same as described earlier(1). Three diets (Table I) which in our earlier

experiments produced fatty livers at 25° were used. Choline and inositol were omitted from the vitamin mixture so that the lipotropic factors of the diets were supplied entirely by the methionine of the dietary casein. At the end of the appropriate periods, as described below, the animals were sacrificed and the livers were removed, weighed, and analyzed for total lipids essentially as described by Dury and Treadwell(4). Twenty-two or 23 rats were placed on each of the 3 diets for 21 days at 25°. At the end of this first period 7 rats on each diet were sacrificed. Those remaining on each diet were divided into 2 groups and maintained on the same diet for an additional 28 days; during this second period, one group was continued at 25°, the other group was kept at 1°. At the end of this 28-day period all rats were sacrificed and the livers analyzed for total lipids. This experimental design provided an evaluation of the curative effect of cold (level of liver lipids after 21 days at 25° compared with that after additional 28 days at 1°) and confirmation of our earlier results on the preventive

TABLE I. Experimental Diets.

| Constituents | Diets, g/100 g | | |
|---------------|----------------|-------|-------|
| | No. 1 | No. 2 | No. 8 |
| Casein* | 5 | 10 | 20 |
| Salt mixture† | 5 | 5 | 5 |
| Cellu flour | 2 | 2 | 2 |
| Starch | 33 | 31 | 16 |
| Sucrose | 33 | 30 | 15 |
| Vitamin mix‡ | 2 | 2 | 2 |
| Lard | 18 | 18 | 38 |
| Cod liver oil | 2 | 2 | 2 |

* Vitamin-free casein.

† Hubbell, Mendel, and Wakeman(5).

‡ For composition see Treadwell *et al.*(1).

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† Several types of fatty livers have been distinguished, principally on mode of dietary production. Fat fatty liver is produced by ingestion of a diet consisting of purified protein, carbohydrate, salts, vitamins, and some easily available form of neutral fat. This type of diet supplemented with sufficient cholesterol produces the cholesterol fatty liver(2).

TABLE II. Curative and Preventive Effects of Cold.

| Diet No. | No. of rats | % survival | Wt change per day, g | Food intake per day, g | Protein efficiency ratio* | Liver, g/100 g body wt | |
|----------------------------|-------------|------------|----------------------|------------------------|---------------------------|------------------------|--------------|
| | | | | | | Wt | Total lipids |
| First period—25°, 21 days | | | | | | | |
| 1 | 7 | 100 | -4 ± .1† | 6.8 ± .5 | -1.2 ± .3 | 6.2 ± .4 | .80 ± .13 |
| 2 | 7 | " | 1.5 ± .2 | 9.0 ± .6 | 1.6 ± .2 | 5.9 ± .4 | .97 ± .22 |
| 8 | 7 | " | 4.0 ± .4 | 9.8 ± .7 | 2.0 ± .1 | 7.0 ± .3 | 1.64 ± .19 |
| Second period—25°, 28 days | | | | | | | |
| 1 | 7 | 100 | .9 ± .1 | 7.1 ± .4 | 2.4 ± .3 | 8.1 ± .6 | 2.10 ± .30 |
| 2 | 7 | " | 1.1 ± .2 | 8.4 ± .6 | 1.3 ± .4 | 6.6 ± .4 | 1.50 ± .16 |
| 8 | 7 | " | 3.6 ± .2 | 11.7 ± .3 | 1.5 ± .0 | 5.3 ± .4 | .98 ± .18 |
| Second period—1°, 28 days | | | | | | | |
| 1 | 9 | 78 | 1.2 ± .1 | 12.7 ± .5 | 1.8 ± .1 | 4.8 ± .2 | .46 ± .05 |
| 2 | 8 | 75 | 1.5 ± .2 | 15.0 ± .5 | 1.0 ± .1 | 4.3 ± .3 | .53 ± .14 |
| 8 | 8 | 100 | 2.6 ± .2 | 15.2 ± .5 | .8 ± .2 | 4.7 ± .1 | .49 ± .06 |

* g change in wt/g protein ingested.

† Mean \pm stand. error.

effect of cold (level of liver lipids at 1° compared with that at 25° after additional 28 days).

Results. The data are summarized in Table II. Individual values are the averages for the number of animals indicated. At 25° the survival was 100% for all 3 dietary groups and for both periods; at 1°, during the second period, there was a decreased survival on the low protein diets 1 and 2. The loss in weight and negative protein efficiency ratio on Diet 1 at 25°, the greater weight gain on Diets 1 and 2 at 1°, and the lower liver weights at 1° are all in agreement with our earlier findings(1). The animals on Diets 1 and 2 which were maintained for the additional 28 days at 25° exhibited increased levels of liver lipids over those sacrificed at the end of the first period. On Diet 8 at 25° the liver lipids were decreased slightly at the end of the second period in comparison to the level after 21 days. In this group the food intake increased and the growth rate and protein efficiency ratio decreased during the second period so that more protein was probably available for lipotropic action which would account for the slight decrease in liver lipids. Comparison of the dietary groups maintained at 25° and at 1° during the second period shows that the cold prevented the increase in liver lipids which occurred at 25°. With all 3 diets the levels of the liver lipids were lower after the additional 28 days at 1° than they were after the initial 21 days at 25°. This finding demonstrates that cold has a curative

effect on the fat fatty liver as well as a preventive one. This curative effect of cold has not been reported previously. It is now apparent that cold exhibits both of the characteristic effects (preventive and curative) of the lipotropic factors choline and methionine. As suggested previously(1) and confirmed by the present data, using the level of liver lipids as a criterion, the rat at 1° has an increased capacity for metabolizing fat even when there is an inadequate supply of dietary lipotropic factors. Cold appears to decrease the dietary requirement for lipotropic factors. Possible explanations for this effect of cold have been discussed earlier(1).

Summary. 1) Three groups of young male rats received one of 3 different choline and inositol-free hypolipotropic diets for 21 days at 25°. Seven rats of each group were then sacrificed. The remainder were continued on the same diet but divided into two subgroups; one was maintained at 1°, the other at 25° for an additional 28 days. The livers of all animals were analyzed for total lipids. 2) Data on food intake, growth rate, protein efficiency ratio, and liver weight confirmed earlier findings. The levels of liver lipids after 28 days at 1° were lower in the 3 dietary groups than in either the comparable groups at 25° after the initial 21 days or after the following 28 days. The results demonstrate that cold has a curative effect on the fat fatty liver and confirm earlier observations on the preventive effect of cold.

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Host Resistance in Hemorrhagic Shock. XIV. Induction of Shwartzman Reaction by Shock Plasma and Tissues.* (23767)

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The plasma of an animal in hemorrhagic shock contains a toxin which is not present in normal plasma(1). Certain properties of this toxin, shared with bacterial endotoxins, are: 1. Ability to reduce the phagocytic activity of normal phagocytes and macrophages *in vitro*(2). 2. Induction of "flight of leukocytes," *i.e.* granulocytopenia with segregation of granulocytes, primarily in capillaries of the lung, liver, and spleen(3). 3. Production of a characteristic febrile reaction in trained rabbits, with development of resistance to the pyrogen following repeated daily administration(4). 4. Development in such resistant animals of tolerance of prolonged hemorrhagic shock, with recovery following a transfusion which is not curative in non-resistant animals (4). The presence of this toxin in shock plasma is not simply an incidental abnormality, for it has been shown to be responsible for the failure of animals in prolonged shock (6 hours) to respond to transfusion; and it is capable of converting reversible shock, in which transfusion of normal plasma leads to full recovery, into a state of shock which is not responsive to any therapy(1). The thesis that this toxin is of bacterial origin stems from two observations: 1. Demonstration, already referred to, that resistance to hemorrhagic shock, induced by making animal re-

sistant to a known bacterial endotoxin, is also induced by the toxin in shock plasma(4). 2. Finding that broad-spectrum non-absorbable antibiotics, administered orally so as to nearly sterilize the gastro-intestinal tract, prevent prolonged shock from becoming irreversible to transfusion, and render blood in prolonged shock free of the toxin(1,5). Two further lines of investigation into the properties of this toxin are presented in this paper to provide additional evidence that the circulating toxin in shock is similar to, or identical with, bacterial endotoxin. The first is the demonstration that the plasma and tissues of the shocked animal elicit a generalized Shwartzman reaction under conditions that may be considered virtually specific for endotoxin(6), while normal plasma and tissues, under identical test conditions, fail to do so. The second is the observation that this activity of shock plasma and tissues resides within a polysaccharide fraction extractable with trichloroacetic acid, in the manner employed by Boivin and Mesrobian(7) for the isolation and purification of bacterial endotoxins.

Method. Hemorrhagic shock was induced in dogs and rabbits, which were exsanguinated after the shock was shown to be irreversible to transfusion(5).

The blood from rabbits was drawn with sterile precautions into a heparinized syringe, and centrifuged in a refrigerated sterile container. Ten ml of the plasma was then injected intravenously into young (1 kg) rab-

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bits prepared for the Schwartzman reaction by an intravenous injection of 0.03-0.06 mg of *E. coli* endotoxin (Difco #1027-B8) given 18 hours earlier. Plasma from normal rabbits was prepared and tested in the same way.

The blood from dogs was drawn into clean glassware, quickly cooled, and the plasma separated in a refrigerated centrifuge. The cooled heparinized dog plasma was diluted with an equal volume of 0.85% sterile saline or distilled water, and deproteinized by the addition of 16 ml of 30% trichloroacetic acid (w/v) per 100 ml of diluted plasma (0.25 N TCA). The suspension of denatured protein was allowed to stand at 4°C for 4 to 16 hours, centrifuged in the cold, and the supernatant fluid dialyzed at 4°C against frequent changes of large volumes of distilled water for 3 to 4 days. The dialyzed extract was then concentrated to approximately 1/100th the starting volume by evaporation at 50-55°C under reduced pressure (4-6 mm Hg).[†] One- to three-tenths of an ml of the concentrated extract was injected intradermally into adult rabbits (2-3 kg) prepared for the Schwartzman reaction by an intravenous injection of 3 ml/kg of 25% sterile colloidal thorium dioxide suspension given 6 hours earlier.[‡] Extracts which failed to produce a Schwartzman reaction when given intradermally were retested by intravenous injection. Tissues from normal and shocked rabbits were homogenized in sterile saline, diluted to a concentration of 20 mg/ml, and centrifuged to obtain a nearly

TABLE I. Generalized Shwartzman Reaction in Endotoxin-Primed Rabbits Challenged with Plasma.

| No. of animals | Priming dose,* intrav. | Challenge dose, intrav. | Response | |
|----------------|---------------------------|-------------------------|----------|------|
| | | | Pos. | Neg. |
| 4 | .03 mg/kg | 20 ml normal plasma | 0 | 4 |
| 6 | .03 " | 20 ml shock plasma | 5 | 1 |
| 4 | .06 " | <i>Idem</i> | 3 | 1 |
| 3 | 2 ml shock plasma | 10 ml shock plasma | 2 | 1 |

* *E. coli* endotoxin (Difco #1027-8B) admin. 18 hr before challenge.

clear saline extract. Three-tenths of an ml of the saline tissue extract was given intravenously to young rabbits (1 kg) prepared for the Schwartzman reaction by an injection of 0.03-0.06 mg of the *E. coli* endotoxin 18 hours earlier. Trichloroacetic acid extracts were prepared from aliquots of whole tissue homogenate suspensions from normal and shocked rabbits, in the manner described for plasma. The concentrated extracts were adjusted to represent the TCA-soluble fraction of 200 mg of fresh wet tissue per ml. These extracts were tested in adult rabbits primed for the Schwartzman reaction with Thorotrast as described above. All recipient animals were killed and examined 48 hours after the challenge or at death, whichever occurred sooner, for gross and microscopic evidence of the Schwartzman reaction. The tissues were fixed in 10% neutral formalin and stained with phloxin and hematoxylin for histologic examination.

Results. None of four rabbits primed with endotoxin and challenged with normal rabbit plasma (Table I) showed any of the gross or microscopic features of the generalized reaction described by Schwartzman(8). In contrast, 8 of 10 rabbits primed with endotoxin and challenged with shock plasma (Table I) developed gross and/or microscopic lesions of the generalized Shwartzman reaction. In addition, 2 of 3 rabbits given a small priming dose of shock plasma (2 ml) in place of endotoxin exhibited the generalized Shwartzman reaction following a challenge dose of shock plasma (Table I).

The saline extracts of liver, lung and spleen

[†] Use of concentrated extract of dialyzed trichloroacetic acid soluble fraction, in place of whole plasma or saline tissue extracts, in addition to providing evidence on the chemical nature of the toxic agent, permits testing of material from differing species without concern for protein-protein incompatibilities between species, and provides much greater flexibility of dosage and sensitivity of detection of the toxin.

[‡] Thorotrast was substituted for *E. coli* endotoxin in preparation of these animals for the Schwartzman reaction because it appears to be capable of inducing a greater degree of sensitivity and regularity of response to known Shwartzman-inciting reagents(9). Thorotrast provides the additional advantage of uniform potency, in contrast to endotoxins, which require biological standardization of each preparation, and which occasionally lose potency on storage(10).

TABLE II. Generalized Shwartzman Reaction in Endotoxin-Primed Rabbits Challenged with Saline Extracts of Rabbit Tissue.*

| Challenge tissue | Response | | | |
|------------------|---------------------|------|--------------------|------|
| | Normal tissue extr. | | Shock tissue extr. | |
| | Pos. | Neg. | Pos. | Neg. |
| Lung | 1? | 5 | 4 | 2 |
| Liver | 0 | 6 | 5 | 1 |
| Spleen | 0 | 6 | 2 | 4 |

* 0.06 mg *E. coli* endotoxin (Difeo #1027-8B) admin. 18 hr before challenge. Two of the 6 animals challenged with saline extract of shock lung died, one of them too soon to develop the Shwartzman reaction.

of 6 normal and 6 shocked rabbits were each tested in endotoxin-primed rabbits. All recipients of normal tissue extract survived and, with the exception of one animal, showed no gross or microscopic signs of the Shwartzman reaction. The one exception, which had received a lung extract, appeared normal on gross examination,⁵ but revealed moderate pulmonary congestion and a few petechial hemorrhages in the kidney on microscopic examination. Two of 18 recipients of saline extracts of shocked tissue died, one within 4 hours of the challenge dose, without the development of gross or microscopic changes of the Shwartzman reaction. Ten of the 16 survivors, and the second non-survivor, demonstrated the generalized Shwartzman reaction (Table II).

Typical *positive* findings at post-mortem examination were (1) heavy, edematous, lungs with numerous discrete areas of hemorrhagic extravasation; (2) suffusion of the liver with blood; (3) petechiae and/or interstitial hemorrhages in the kidneys; (4) focal necrosis on the capsular and cut surface of the kidneys; and, in a few instances, (5) intramural hemorrhages in the small and large intestine. In some instances organs appeared to be within normal limits on gross inspection, but revealed typical changes on microscopic examination.

In general, the microscopic examination simply confirmed the gross findings of congestion, interstitial hemorrhage, and focal necrosis. In addition, however, there was diffuse infiltration of tissues with granulocytes, particularly in the lungs; and some of the

renal glomeruli were occluded with a homogeneous acidophilic substance resembling fibrin.

The concentrated polysaccharide fraction of the plasma of 13 shocked dogs and of 6 normal dogs was tested by intradermal injection in Thorotrast-primed rabbits. Four of 13 recipients of the shock plasma extract died, while none of the 6 recipients of normal plasma extract succumbed. Ten of the 13 animals tested with shock plasma extract showed gross and microscopic signs of the Shwartzman reaction (Table III). All 6 animals tested with normal plasma extract were free of the stigmata of the Shwartzman reaction. All extracts giving a negative response on intradermal injection were retested *via* intravenous administration, with the result that one of the 3 negative shock plasma extracts gave a positive response, and all 6 normal plasma extracts remained totally inactive.

The 4 rabbits killed by the shock plasma extract exhibited hemorrhagic peritoneal fluid and a large retro-peritoneal and/or peri-renal hemorrhage, in addition to the typical gross lesions of the Shwartzman reaction. The same occurred to a lesser degree in 5 of the 9 Shwartzman-positive survivors.

The concentrated polysaccharide extract of tissues (liver, lung, kidney, and skeletal muscle) of irreversibly shocked rabbits and of normal rabbits was injected intravenously into Thorotrast-primed rabbits in doses equivalent to 3 to 10 times the amount of whole tissue represented by the saline extracts (Table IV). Extracts prepared from normal rabbit tissues were uniformly non-toxic, and failed to induce the Shwartzman reaction. Extracts prepared from irreversibly shocked rab-

TABLE III. Generalized Shwartzman Reaction in Thorotrast-Primed Rabbits Challenged with 0.3 ml Trichloroacetic Acid Extract Concentrate* of Plasma.

| No. of animals | Challenge material | Response | |
|----------------|-------------------------------|----------|------|
| | | Pos. | Neg. |
| 6 | Ext. normal plasma intraderm. | 0 | 6 |
| 6 | <i>Idem</i> intrav. | 0 | 6 |
| 13 | Ext. shock plasma intraderm. | 10 | 3 |
| 3 | <i>Idem</i> intrav. | 1 | 2 |

* 0.3 ml concentrate equivalent to 20 ml of plasma.

TABLE IV. Generalized Schwartzman Reaction in Thorotrast-Primed Rabbits Challenged with 0.3 ml Trichloroacetic Acid Extract Concentrate* of Tissue.

| Challenge tissue | Response | |
|------------------|----------|-------|
| | Normal | Shock |
| 2 lung | 0 | + |
| 2 liver | 0 | + |
| 2 kidney | 0 | + |
| 2 muscle | 0 | ? |

* 0.3 ml concentrate equivalent to extractable polysaccharide from 60 mg fresh tissue. The data in this table are from 2 normal and 2 shocked rabbits.

bits, with the exception of skeletal muscle extract, produced the generalized Schwartzman reaction in each instance, and 2 of 6 animals died. In the case of the skeletal muscle extract, the results are still inconclusive.

Discussion. The induction of the generalized Schwartzman reaction by a challenge dose of plasma, saline tissue extract, or a partially purified polysaccharide fraction of the plasma or tissues of shocked animals, demonstrates that the toxin present in these preparations is bacterial endotoxin. This statement is based on the prevailing view that, whereas a number of high molecular weight substances other than bacterial endotoxin are capable of *preparing* an animal for the Schwartzman reaction, and of *inducing* a *localized* Schwartzman reaction, only *bacterial* endotoxin has, to date, been shown to be capable of inducing the generalized Schwartzman reaction(6,8).

Recently Landy and Shear stated that the biological properties of endotoxin are not specific for bacterial polysaccharides, since polysaccharide fractions from the tissues of normal mice, isolated by tryptic digestion and trichloroacetic acid-phenol extraction, exhibit the same properties as bacterial endotoxin, except that they are less potent(10). Such tissue polysaccharides are said to "manifest endotoxic activities only when they are released from their native state of firm combination with other cellular components"(10). The endotoxic properties consisted of a lethal effect, a pyrogenic effect, a tumor necrotizing effect, and a preparative activity for the local Schwartzman reaction. But a generalized Schwartzman reaction has been difficult to

elicit. Several facts are in conflict with the suggestion that these polysaccharides rather than bacterial endotoxins may be the source of the toxin in the shocked animal. First, the oral administration of non-absorbable antibiotics prior to the induction of shock renders an animal tolerant to hemorrhagic shock, and prevents the appearance of the toxin in the plasma(1). It is unreasonable, in our view, to postulate that non-absorbable antibiotics might have an action upon the host cells so as to prevent the release of tissue polysaccharides that Landy and Shear imply may be released in shock(10). Moreover, the lack of effect of antibiotics upon the course of hemorrhagic shock when the intestinal flora are resistant to the antibiotics points again to the bacteria, not to the host tissue, as the source of the toxin. Second, the fact that endotoxic activity is present in the plasma long before shock becomes irreversible, and can be demonstrated after as little as 5 minutes of hemorrhagic shock(11), is equally difficult to reconcile with the thesis of a toxic tissue polysaccharide. The conventional procedures for the isolation of polysaccharides, which are less drastic than that of Landy and Shear, fail to demonstrate toxic polysaccharides in normal tissue, but do demonstrate toxic polysaccharide in shock tissue. If the thesis of Landy and Shear is correct, the degree of tissue injury equivalent to homogenization, tryptic digestion, and trichloroacetic acid-phenol extraction, used by them to demonstrate toxic polysaccharide, would have to result from an exposure to as little as 5 minutes of hemorrhagic shock. This is far more severe tissue damage than other observations would indicate for such a short exposure to hemorrhagic shock. On the other hand, relatively minor differences in the methods of isolation and purification of endotoxin may alter the antigenic and/or toxic properties of polysaccharides.

Zweifach and Thomas state that the development of tolerance to shock following induced tolerance to endotoxin may represent a non-specific enhancement of R.E. function, and does not necessarily imply that the cross-tolerance to shock is specifically related to an increased capacity to destroy endotoxin *per se*

(11). It is, of course, possible that stress, in some undefined way, may induce a non-specific enhancement of R.E. function; but such a hypothesis will not explain the observation that a circulating toxin in the shocked animal, which can convert reversible to irreversible shock, and which can induce tolerance to shock, is absent in shocked animals rendered tolerant to shock by pre-treatment with antibiotics, or by repeated prior exposure to shock plasma or endotoxin. In support of their hypothesis that non-specific resistance is involved in shock they report data purporting to show that while endotoxin can induce cross tolerance to shock from drum trauma, the reverse is not the case; for they state that sub-lethal drum trauma fails to induce cross-tolerance to endotoxin(11). A statistical examination of their data, however, indicates that sub-lethal drum trauma *does* induce significant cross-tolerance to endotoxin. Since the cross-tolerance so demonstrated does not proceed via classic immunochemical mechanisms, one cannot regard it as constituting adequate proof of the *chemical* identity of the shock toxin and endotoxin. Nevertheless, the demonstration of cross-tolerance in both directions (*i.e.* trauma/endotoxin and endotoxin/trauma) is best explained on the theory that endotoxemia is a concomitant of shock.

Summary. It has been demonstrated that whole plasma and saline extracts of lung, liver, spleen, and kidney of the shocked animal, but not the normal animal, are capable of inducing the generalized Schwartzman reaction in endotoxin or Thorotrast-primed re-

cipients with the same severity and frequency as purified bacterial endotoxin. The active material may be isolated by extraction with trichloroacetic acid and dialysis in the manner employed for bacterial polysaccharides. A number of similarities are noted in the biologic effects of shock toxin and bacterial endotoxin. The appearance of the toxin in the circulation within minutes of the onset of shock and the prevention of its appearance by antibiotic pretreatment are discussed with respect to the endogenous or exogenous source of the toxin.

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A Vulnerable and Rate-Limiting Step in Urea Synthesis in Patients with Hyperammoniaemia.* (23768)

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The role of portal-systemic shunts, either spontaneously developed or surgically constructed, in the production of hyperammoni-

aemia and ammonia intoxication is well established(1,2,3) but there has been no direct evidence regarding vulnerability in man of the mechanism of synthesis of accumulated ammonia to urea. Animal experiments, how-

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ever, have suggested that there may be a rate-limiting step in the Krebs-Henseleit ornithine cycle. Gornall and Hunter(4) showed that during incubation of rat liver slices, citrulline accumulated in the medium. Archibald(5) found that when he produced hemorrhagic shock in dogs, there was a 2-5 fold increase in plasma citrulline, a simultaneous depletion of circulating arginine, and depression of urea formation. Both of these studies suggested that conversion of citrulline to arginine may be a rate-limiting and vulnerable point in urea synthesis.

This study was designed to demonstrate whether or not such a specific point of breakdown in urea synthesis exists in patients with liver disease and hyperammoniaemia. This was done by analyzing peripheral venous blood from patients on the wards, for citrulline, arginine and ammonia.

Methods. Citrulline was measured by modification of the method described by Archibald(5). 0.57 ml of 2% Sigma urease powder was added to 4 ml of plasma for digestion. The digest was dialyzed against 13.5 ml of 0.02 N H_2SO_4 to obtain a larger volume to pass through the resin column containing amberlite IR-120 (H), analytical grade.[†] Resin columns were washed with 15% NaCl solution. The resin was then converted to the hydrogen form with several 10 ml washes of 3 N HCl. The final dialysate represented a 5-fold dilution of plasma. The colorimetric values were obtained as described by Archibald(5). Arginine was determined by performing the Sakaguchi reaction as described by Sims(6) on 4 ml of Folin-Wu blood filtrate. It has been shown by Weber(7) that 95% of the color which results when the Sakaguchi reaction is performed on blood filtrates is due to arginine. A standard curve using citrulline and arginine solutions of 4 different concentrations was run simultaneously with every citrulline and arginine determination. Ammonia was determined by modification of the microdiffusion technic of Conway(1).

Results. Fig. 1 shows scattergrams of

CITRULLINE



FIG. 1. Plasma citrulline levels in normal individuals and in patients with disorders of liver and portal circulation. Cross-bars represent calculated mean.

plasma citrulline concentrations in 3 groups of patients: (1) normal, (2) patients with liver disease and blood ammonia levels greater than $70 \mu g$ ammonia N/100 ml, (3) patients with surgically constructed portal-systemic shunts. Fifteen normal persons had an average plasma citrulline level of $9.2 \mu g/ml$, range of $6.3-12.5 \mu g/ml$. Sixteen determinations of citrulline on patients with various types of liver disease and blood ammonia-nitrogen levels greater than $70 \mu g/100$ ml revealed an average plasma citrulline of $18.4 \mu g/ml$, range $10.0-27.5 \mu g/ml$. This difference is statistically highly significant, with a p value of less than 0.001. Five determinations of citrulline levels in patients with surgically constructed shunts showed average citrulline level of $16.2 \mu g/ml$, range of $11.3-21.6 \mu g/ml$.

Fig. 3 shows the relation between ammonia and citrulline levels in peripheral blood. The correlation coefficient of 0.66 with standard error of 0.18 confirms the relationship between the ammonia load and elevations in citrulline levels.

[†] Supplied through courtesy of Rohm & Haas Co., Washington Sq., Phila., Pa.

ARGININE

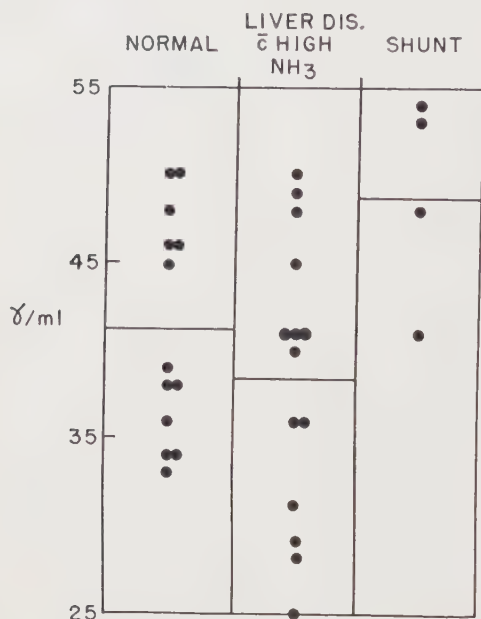


FIG. 2. Blood arginine levels in normal individuals and in patients with disorders of liver and portal circulation. Cross-bars represent calculated mean.

Fig. 2 is a scattergram of blood arginine concentrations in the same groups, although a few patients did not have both arginine and citrulline levels determined simultaneously. The average blood arginine level in 13 normal people was $41.2 \mu\text{g/ml}$, range $32.5\text{--}50.0 \mu\text{g/ml}$. Fourteen determinations on patients with liver disease showed an average blood arginine of $38.6 \mu\text{g/ml}$, range of $25\text{--}50 \mu\text{g/ml}$.

Four determinations on patients with surgical shunts gave an average value of $48.8 \mu\text{g/ml}$, range of $41.3\text{--}53.7 \mu\text{g/ml}$. This is considerably higher than the mean level found in normals and in unoperated cirrhotics. The number of cases is too small to calculate statistical probabilities but the possible reasons for elevated levels in patients with shunts will be discussed later.

All patients reported in this series and their citrulline, arginine and ammonia levels are recorded in Table I.

Discussion. The results of this study show that plasma citrulline levels are increased in patients with hyperammoniaemia and liver disease, the average level in the pathologic

state being double that in normal individuals. Since citrulline is a small, readily diffusible molecule, does not enter into protein structure, is not a part of dietary intake and within limits of our present knowledge, is synthesized in the body only by enzymatic action on ornithine in the liver, it may be assumed that this citrulline measured in the peripheral blood is a direct reflection of citrulline metabolism in the liver. The fact that citrulline accumulates suggests that, the rate of conversion of citrulline to arginine has slowed relative to rate of production of citrulline from ornithine. Conversion of citrulline to arginine is therefore, the site of a relative block in urea synthesis in the abnormal liver in man.

Blood arginine values are somewhat more difficult to evaluate than the plasma citrulline levels because substantial amounts of arginine are absorbed from dietary protein and because arginine, unlike citrulline, is an active participant in many facets of body metabolism. The level of arginine in peripheral venous blood is then, a balance between (1) arginine absorbed by the gut from dietary protein, (2) liver synthesis of arginine, (3) peripheral utilization of arginine for synthesis of protein tissue structure, (4) degradation of tissue protein releasing arginine into the amino acid pool and (5) degree of shunting of portal blood around the liver.

Despite the elevations in citrulline (Table

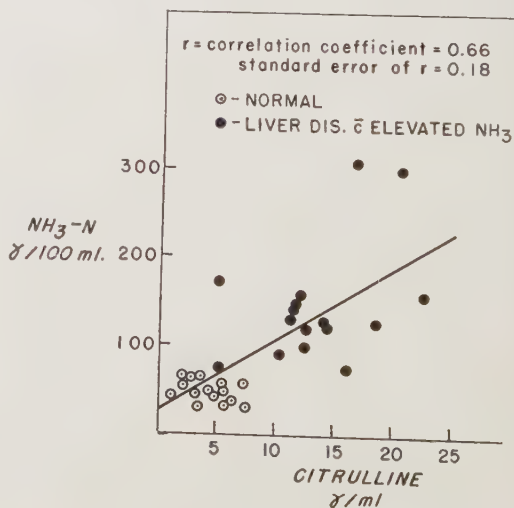


FIG. 3. Correlation between blood ammonia and plasma citrulline levels.

TABLE I. Blood Ammonia, Blood Arginine and Plasma Citrulline Levels in Patients with Disorders of the Liver and Portal Circulation.

| Patient No. | NH ₃ , mg/100 ml | Citrulline, mg/ml | Arginine, mg/ml | Diagnosis |
|-------------|--------------------------------|----------------------|--------------------|-------------------------------|
| 1 | 309 | 21.6 | | Infectious hepatitis |
| 2 | 129 | 19.1 | | Carcinoma metastatic to liver |
| 2 | 170 | 10.0 | 41.2 | <i>Idem</i> |
| 3 | 156 | 16.9 | | Laennec's cirrhosis |
| 4 | 142 | 16.6 | 41.2 | <i>Idem</i> |
| 5 | 91 | 15.3 | 36.2 | " |
| 5 | 74 | 10.3 | 27.5 | " |
| 5 | 121 | 17.5 | 31.2 | " |
| 6 | 133 | 16.3 | 40.0 | " |
| 7 | 122 | 19.4 | 45.0 | " |
| 8 | 160 | 27.5 | 50.0 | " |
| 9 | 143 | 16.6 | 28.8 | " |
| 10 | 96 | | 25.0 | " |
| 11 | 75 | 21.0 | 36.2 | " |
| 12 | 101 | 17.5 | 48.8 | Biliary cirrhosis |
| 12 | 136 | 23.4 | 41.3 | <i>Idem</i> |
| 12 | 299 | 25.6 | 47.5 | " |
| 13 | 182 | 13.7 | | Porta-caval shunt |
| 13 | 121 | | 53.7 | <i>Idem</i> |
| 13 | 115 | 21.6 | 47.5 | " |
| 14 | 134 | 19.7 | | Spleno-renal shunt |
| 15 | 151 | 11.3 | 52.5 | Porta-caval " |
| 15 | 114 | 14.7 | 41.3 | <i>Idem</i> |

I, Fig. 1), there is no significant change in arginine levels in the unoperated patients with cirrhosis. These observations do not of necessity negate the possibility of a rate-limiting step in the Krebs-Henseleit cycle with decreased formation of arginine from this source. As mentioned in the above paragraph, other sources, particularly absorption of arginine from the gastro-intestinal tract by-passing the liver via portal-systemic collaterals, would tend to maintain the level of arginine in the peripheral blood. This possibility is supported by the high levels of arginine found in patients with surgically constructed portal-systemic shunts.

Further studies on this defect in urea synthesis are in progress.

Summary. 1. Elevations of citrulline in blood of patients with hyperammoniaemia and cirrhosis of the liver have been observed. 2. On the basis of the reported observations, a rate-limiting and vulnerable step in urea synthesis in man is suggested.

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Effects of Excess Dietary Methionine and Niacinamide in the Rat. (23769)

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Excess dl-methionine in the diet inhibits growth and causes a marked loss of body fat, enlargement of the kidneys, increased excretion of creatinine and a small increase in liver fat in the immature rat (1,2,3). Excess dietary niacinamide also inhibits growth in the rat (4). Since these effects were observed in short term studies in rats maintained on basal diets, this study was designed to ascertain the effects of prolonged administration of excess dietary methionine and niacinamide on the growth rate and organ weights of rats maintained on a standard laboratory ration.

Methods. The effect of various dietary supplements on growth in the rat was determined in 2 series of experiments. In the first series, 5 groups of 12 male Holtzman rats, weighing 110-135 grams, were treated with various combinations of dl-methionine, niacinamide, and biotin for a period of 12 weeks. The rats were allowed food and water *ad libitum*. The food a standard rat chow, was finely ground and definite amounts of the nutrients being tested were added. The animals were weighed at weekly intervals and 4 rats of each group were sacrificed at the end of 2, 8 and 12 weeks of treatment. The adrenal glands, liver and kidneys were removed and weighed. In the second series, 4 groups of 10 rats were administered dietary supplements of malic acid, dl-methionine and niacinamide. The procedures used were the same as above except animals were sacrificed only at the end of 8 and 12 weeks of treatment. The results obtained from the 2 series of experiments were analyzed statistically using the Analysis of Variance Method. For this analysis all gland weights were expressed as a function of the body weight.

Results. All diets containing excess methionine inhibited the growth rate during the first 2 weeks of treatment ($P < 0.01$) (Fig. 1). At the end of 8 and 12 weeks of treatment,

only the animals receiving diets containing both 2% methionine and 0.1% niacinamide exhibited a significant retardation of growth ($P < 0.01$). However the addition of 1% malic acid to the diet prevented the decrease in the growth rate. Niacinamide administered at the level of 0.1% of the diet for 8 weeks caused no significant change in the growth rate, but at the 0.2% level, growth was enhanced and at the 0.4% level, growth was inhibited ($P < 0.05$) (Fig. 2). During the next 4 weeks of treatment the body weights of animals receiving 0.2% niacinamide in the diet returned to control levels but inhibition of growth by the 0.4% dosage became more marked ($P < 0.05$). As shown in Table I, there is a transient enlargement of the kidneys during the first few weeks of treatment with all diets containing 2% methionine. In addition, a transient adrenal hypertrophy is observed in rats treated with methionine alone. There is a decrease in the liver size in rats receiving 2% methionine plus 0.1% niacinamide for 12 weeks but this is prevented by the ad-

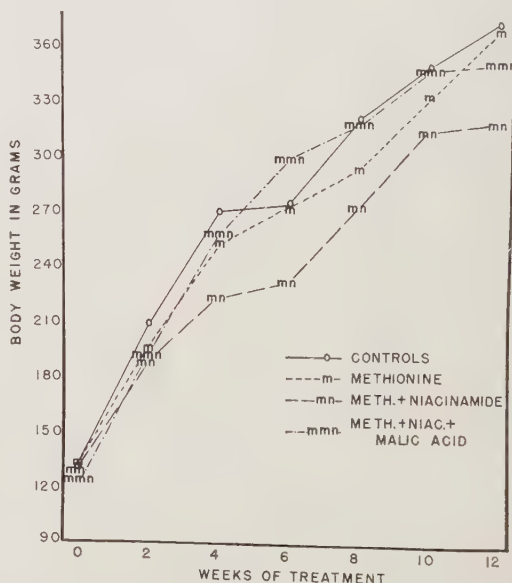


FIG. 1. Effect of excess dietary methionine, niacinamide and malic acid on growth rate of the rat.

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TABLE I. Effect of Excess Dietary Nutrients on Relative Weights of Liver, Kidney and Adrenal Glands of Rats.

| Treatment† | Liver (g %)* | | | Kidney (g %) | | | Adrenals (mg %) | | |
|--------------------------|--------------|------|-------|--------------|-----|------|-----------------|-------|------|
| | | | | | | | | | |
| | 2 | 8 | 12 | 2 | 8 | 12 | 2 | 8 | 12 |
| Control | 4.73 | 3.92 | 3.92 | .96 | .79 | .79 | 17.0 | 15.6 | 13.9 |
| 2% M | 5.04 | 3.74 | 4.10 | 1.17§ | .83 | .77 | 20.0‡ | 15.9 | 12.9 |
| 2% M .1% N | 5.04 | 3.77 | 3.39§ | 1.12§ | .84 | .83 | 18.2 | 16.9 | 12.4 |
| 2% M .1% N .004% B | 5.29 | 3.95 | 3.96 | 1.13§ | .82 | .80 | 17.5 | 14.6 | 13.2 |
| 2% M .1% N 1% MA | | 3.89 | 3.55§ | | .87 | .85 | | 13.7 | 13.1 |
| 2% M 1% MA | | 4.02 | 3.84 | | .89 | .90 | | 15.8 | 13.6 |
| .1% N | 4.84 | 3.67 | 3.82 | 1.03 | .79 | .69‡ | 15.2 | 15.1 | 12.9 |
| .2% N | | 4.06 | 3.63‡ | | .74 | .74 | | 13.9 | 13.0 |
| .4% N | | 4.10 | 3.67 | | .73 | .84 | | 17.4 | 15.5 |
| .004% B | 4.92 | 3.62 | 3.77 | .99 | .77 | .81 | 17.0 | 12.0§ | 12.2 |
| 1% MA | | 3.83 | 4.05 | | .80 | .83 | | 14.7 | 13.7 |

* Gland wt/100 g of body wt.

† M = Methionine; N = Niacinamide; B = Biotin;

MA = Malic acid.

‡ Significant at the 5% level.

§ Significant at the 1% level.

dition of 0.004% biotin to the diet. The relative weight of the adrenal glands in rats receiving 0.004% biotin only is significantly decreased after 8 weeks ($P < 0.01$) but not after 12 weeks of treatment.

Discussion. Handler and Perlzweig(5) suggested that excess dietary niacinamide

causes a retardation of growth by inducing a secondary methionine deficiency. Niacinamide undergoes obligatory methylation prior to excretion and methionine appears to be the source of the labile methyl groups. The data presented here are not in agreement with this hypothesis since niacinamide was found to augment the effect of methionine on the growth rate rather than to inhibit it. However, the discrepancies between the data presented here and that obtained by Handler and Perlzweig are probably caused by the difference in the dosages and the basal diets that were used. The prevention of the effect of treatment with methionine and niacinamide on growth by malic acid suggests that growth is retarded *via* an action on carbohydrate metabolism at or preceding the tricarboxylic acid cycle. Since pyridoxine will counteract the effect of moderate amounts of methionine on growth in the rat(6), the addition of malic acid to the diet may compensate for a pyridoxine deficiency. It is interesting to note that malic acid prevents the inhibition of growth but not the decrease in liver size caused by the treatment whereas the reverse is seen when biotin is added to the diet. In general, the various

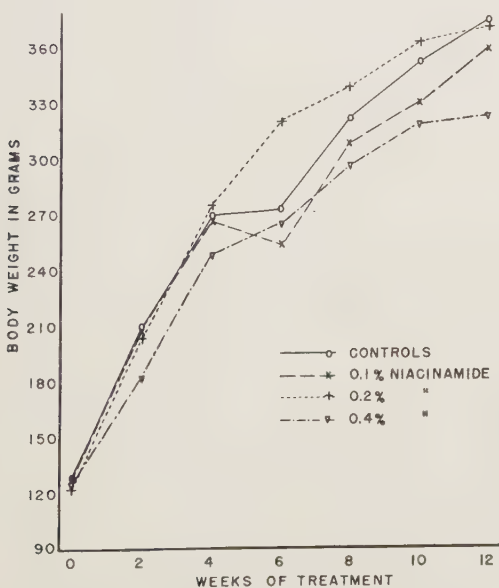


FIG. 2. Effect of various dosages of niacinamide on growth rate of the rat.

treatments are relatively non-toxic. There is a transient hypertrophy of the kidneys during the first few weeks of treatment with methionine but as prolonged treatment did not cause adrenal hypertrophy, it is doubtful that any of the regimens placed a severe stress on the animals.

Summary. The effects of prolonged administration of excess dietary dl-methionine and niacinamide on growth rate and on the relative weights of the liver, kidneys and adrenal glands of rats were determined. Although both methionine and niacinamide caused a decrease in the growth rate, a greater retardation of growth was obtained when both nutrients were administered together. The addition of 1% malic acid to the diet prevented the inhibition of growth by these substances.

Although methionine caused a transient enlargement of the kidneys, none of the treatments appeared to place a severe stress on the animals.

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Prevention of Aortic Atherosclerosis in Rabbit by Intravenous Microcrystallized Estradiol Benzoate and Dextran®. (23770)

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It has been demonstrated that estrogens are able to prevent development of atherosclerosis in several species(1-3) and exert a beneficial effect even in man (see review in 4). Experiments designed to reproduce these results in male rabbits, however, have been consistently negative (personal communication) although contradictory conclusions have been reported in work done with female rabbits(5,6). In the present paper prevention of aortic atherosclerosis in cholesterol-fed male rabbits by a new method, *i.e.*, by the intravenous injection of a water-insoluble estrogen, is reported.

Methods. In this study male rabbits of mixed breed weighing 1.5 to 2.5 kg were maintained on carrots, clover, water and additional vitamins. All individuals, except the controls, also received 6 days a week 750 mg

of crystalline cholesterol in 5 ml of sunflower seed oil (I. N. 130) thoroughly mixed with mashed carrots. One week after starting the cholesterol feeding, half of the animals thus fed were injected intravenously twice a week with 4 ml of saline in 2 divided doses, 2 hours apart; the other half received intravenously twice a week 2 ml of 6% Dextran® and 0.5 mg of microcrystallized estradiol benzoate† in 5 ml of saline, also in divided doses given 2 hours apart. Microcrystals less than 1 μ in diameter and suitable for intravenous

TABLE I. Aortic Macroscopic Atherosclerosis in Male Rabbits.

| Group | No. of rabbits | Lesion grade | | | | |
|---------------------|----------------|--------------|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 |
| Control | 12 | 12 | 0 | 0 | 0 | 0 |
| Chol-saline | 21 | 6 | 4 | 4 | 6 | 1 |
| Chol-dext-estradiol | 22 | 14 | 1 | 5 | 2 | 0 |

Chol, cholesterol; dext, Dextran®; estradiol, estradiol benzoate.

† Estradiol-3-benzoate (Schering).

* The Research Department is partly supported by Asociación Cardiológica Inchauspe, aided by grant from Asociación Argentina de Compañías de Seguro.

TABLE II. Percentage Distribution of Aortic Atherosclerosis in Cholesterol-Fed Rabbits.

| Group | No. of rabbits | Without lesions | % | With lesions | % | "t" | p |
|---------------------|----------------|-----------------|------|--------------|------|---------|------|
| Chol-saline | 21 | 6 | 28.6 | 15 | 71.4 | } 2.602 | <.02 |
| Chol-dext-estradiol | 22 | 14 | 63.6 | 8 | 63.3 | | |

"t," Student's "t" test(8); p, probability; chol, cholesterol; dext, Dextran®; estradiol, estradiol benzoate.

injection were freshly prepared by precipitating a concentrated alcoholic solution of estradiol benzoate into normal saline.

Phagocytosis of colloidal thorium has been demonstrated to occur in the atheromatous aorta of rabbits(8) and it is reasonable to suppose that the hormone microcrystals are phagocytized in the same way. It was felt that an even greater phagocytosis by blood vessel intima might be attained if uptake by the spleen were blocked. Therefore Dextran, a macromolecular polymer known to accumulate in the spleen of rabbits(9) was previously injected. Dextran itself has no effect on the development of atherosclerosis in cholesterol fed rabbits (unpublished data).

Results. The controls and 43 animals which survived 60 days of this experimental regime were sacrificed and their aortas examined. The observed macroscopic atherosclerosis was graded on an arbitrary 0 to 4 plus scale. Results shown in Table I demonstrate that the Dextran-estrogen-treated group had considerably less atherosclerosis than the saline-treated group. When the animals were rearranged as positive or negative for atherosclerosis and the conclusions assayed with Student's "t" test for the significance of percentages(7), the results were found to be statistically significant at the level of $p < 0.02$ (Table II). Weight of the testicles and of the hypophyses showed no sta-

tistically significant differences in the three groups of rabbits (Table III).

Discussion. Although reasons accounting for prevention of atherosclerosis in the present experiment have not yet been explored, it is interesting to speculate concerning mechanisms probably involved, especially since similar experiments carried out even with higher doses of estrogens have been thus far consistently negative(5). It is possible that in our experiments, the hormone was more effective either because higher blood levels were obtained and/or because circulating microcrystals were phagocitized by the altered endothelium, thus becoming veritable "intraarterial pellets."

The effects of microcrystallized estradiol in other arterial territories is under study at present; the application in man of this new method of administering drugs with a possible predominant action on altered arterial intima has already been tried in twenty patients without untoward results(10).

Summary. Intravenous administration of microcrystals of estradiol benzoate 2 hours after an intravenous injection of Dextran® prevented aortic atherosclerosis in cholesterol-fed rabbits. The testicle and the hypophysis weights were similar to control groups. A predominant action of the injected drugs in the arterial lesions is suggested.

TABLE III. Weight of Endoerines in Cholesterol-Fed Rabbits.

| Group | No. of rabbits | Testicles (g) | No. of rabbits | Hypophysis (mg) |
|-------------------------|----------------|----------------|----------------|-----------------|
| (1) Control | 11 | 4.582 ± 1.678* | 9 | 21.51 ± 5.15 |
| (2) Chol-saline | 20 | 4.227 ± 1.805 | 15 | 22.8 ± 7.24 |
| (3) Chol-dext-estradiol | 21 | 3.986 ± 1.152 | 16 | 21.3 ± 7.65 |
| "t" 1 vs 2 | | .538 (p < .6) | | .522 (p < .6) |
| " 1 vs 3 | | 1.185 (p < .3) | | .073 (p > .9) |
| " 2 vs 3 | | .507 (p < .7) | | .177 (p < .9) |

* Stand. dev.

Chol, cholesterol; dext, Dextran®; estradiol, estradiol benzoate; "t," Student's "t" test; p, probability.

We wish to express our appreciation to Prof. Blas Moia for generous help during completion of these experiments.

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Effect of Nephrosis on Renal QO_2 and Histochemical Appearance of Cytochrome Oxidase and Succinic Dehydrogenase. (23771)

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Investigation of the appearance and distribution of certain histochemically demonstrable renal tubular enzymes in rats with nephrosis induced by injection of anti-rat kidney-rabbit serum revealed an appreciable depletion of cytochrome oxidase (G-nadi oxidase) and succinic dehydrogenase(1). Certain physiologic and morphologic considerations suggested that these alterations were related to degree of proteinuria rather than to a nephrotoxic effect of the serum employed. It therefore appeared worthwhile to evaluate such enzymatic activity in nephrosis produced by another etiologic agent and to attempt to quantitate any changes if present, manometrically. In addition, this information would also provide an opportunity for correlation of results obtained by the *in vitro* and histochemical staining technics. In the present study nephrosis was induced by appropriate doses of aminonucleoside, an agent which has been demonstrated to produce such a state with relative ease and a high degree of consistency(2,3).

Methods. Forty-two adult female Wistar rats weighing 150-200 g were utilized. Group A consisted of 21 animals that received 0.003 ml of a 0.5% aqueous solution of aminonucleoside* per g of body weight subcutaneously for 9 consecutive days and were sacrificed 5

days after the last injection. There were 8 animals in Group B that received a similar dose of aminonucleoside but were sacrificed after only 6 injections. Group C was comprised of 13 untreated rats of similar sex, strain and weight. Protein content of the urine was determined daily and the serum cholesterol, total lipid and total protein at the time of sacrifice by methods described elsewhere(1). All animals were sacrificed by decapitation. Their kidneys were quickly removed. One was placed immediately in ice after removing its capsule and ureter. Thin slices consisting of the cortex and medulla were prepared according to the method of Deutsch(4). These were incubated in Krebs-Ringer-phosphate-glucose buffer, pH 7.2 at 37°C in a total volume of 3.0 ml. The gas phase consisted of 5% CO_2 and 95% O_2 . Liberated CO_2 was absorbed by 20% KOH placed in the center well. The oxygen consumption was followed from 30 to 60 minutes, after which time the tissues were removed and dried at 108°C for 2 hours. Their dry weights were obtained after desiccation for 18 hours. 0.6 ml of a 0.5% solution of aminonucleoside, previously neutralized and

* Generously furnished by Dr. Stanton Hardy, Lederle Laboratories.

TABLE I. Renal QO₂ of Nephrotic and Normal Rats.

| Group | No. | QO ₂ (ml/mg dry wt) |
|---------------------------|---------|-----------------------------------|
| A. (Treated, nephrotic) | 21 | 14.5 ± 3.0 |
| B. (" , not nephrotic) | 8 | 17.4 ± 2.4 |
| C. (Untreated, ") | 13 | 18.7 ± 1.9 |
| Difference between groups | P value | |
| A & B, A & C | <.01 | |
| B & C | >.1 | |

dissolved in the buffer, was added from a side arm to the kidney slices from 6 additional untreated controls twenty minutes after normal respiration was observed. QO₂ was determined by appropriate calculations and the statistical significance between groups was obtained by utilization of Fisher's "t". The remaining kidney was longitudinally sliced and one-half immediately frozen on dry ice, sectioned at -20°C in a cryostat and stained for the demonstration of cytochrome oxidase utilizing α -naphthol and dimethyl-p-phenylenediamine hydrochloride(5) and succinic dehydrogenase with a substrate of neotetrazolium chloride and sodium succinate(6). The remainder of the kidney was fixed in Zenker's acetic fluid and processed for histologic study in the usual manner.

Results. All of the animals of Group A developed the nephrotic syndrome. Proteinuria, exceeding 100 mg/18 hours first appeared on the 8th day following the initial injection and was followed generally in 2 days by the presence of ascites. Hyperlipemia, hypercholesterolemia and hypoproteinuria were also present at the time of the sacrifice. The microscopic appearance of the renal lesions produced by aminonucleoside will be described in detail elsewhere. On the other hand, those rats which were sacrificed after only 6 injections (Group B) failed to exhibit any chemical, clinical or morphologic manifestations of the nephrotic syndrome.

As indicated in Table I there was a statistically significant decrease of oxygen consumption by the kidney slices from rats with nephrosis (Group A) as compared to those of the untreated controls or those animals sacrificed prior to the onset of the nephrotic state. The addition of aminonucleoside to the kid-

ney slices from normal animals was without statistically significant effect on QO₂ when compared with the internal control values.

Similarly, a depletion of histochemically demonstrable succinic dehydrogenase and cytochrome oxidase (G-nadi oxidase) was apparent only in those animals with the nephrotic state (Fig. 1 and 2). Histochemically, the loss of these respiratory enzymes appeared in those topographic areas corresponding to the greatest concentration of proximal convoluted tubules and ascending limbs of Henle, sites which also exhibited a depletion of mitochondrial elements (unpublished). The degree of depletion noted histochemically usually appeared greater than might be expected from the manometric findings although in many instances changes observed were of the same order of magnitude.

Discussion. Reduction of renal tubular succinic dehydrogenase has been noted following a variety of experimental procedures (7). However, it appears unlikely that the reduction in renal QO₂ and histochemically demonstrable succinic dehydrogenase and cytochrome oxidase (G-nadi oxidase) activity observed in nephrosis induced by aminonucleoside is the result of a direct toxic effect of this agent. The addition of an excessive quantity of this drug *in vitro* failed to significantly affect the QO₂ of fresh kidney slices from normal rats. In addition, no effect on enzymatic activity was apparent in rats receiving aminonucleoside which were sacrificed prior to the onset of nephrotic manifestations. On the other hand, the histochemical findings are identical to those previously noted in animals with nephrotoxic serum nephrosis with massive proteinuria and ascites. Such information suggests that the mechanism responsible for suppression of cellular respiration in the nephrotic state produced by these diverse modalities may be similar and related to the massive proteinuria. Increased tubular reabsorption of protein which also is apparent in both nephrotic states(1), further suggests that such enzymatic depletion may result during an accentuated phase of this tubular activity. Kretchmer and Dickerman(8) have noted a decrease in succinic oxidase and cytochrome oxidase activity in the droplet and

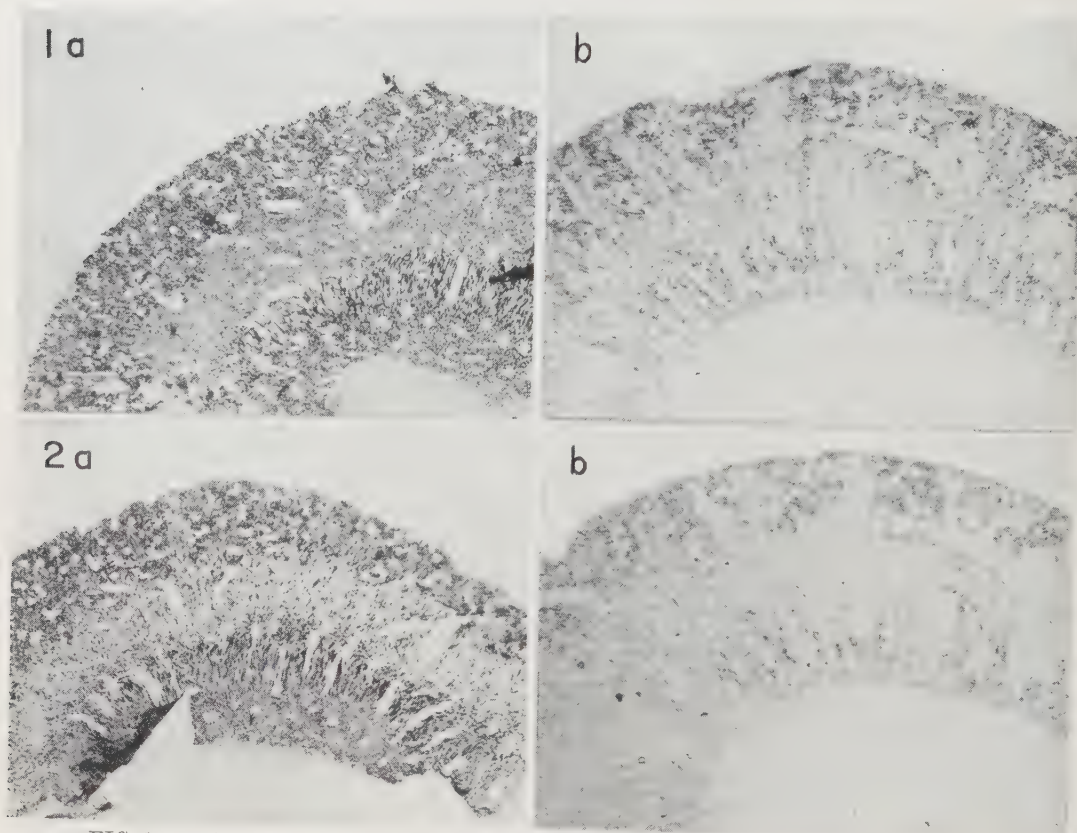


FIG. 1. a. Appearance of renal succinic dehydrogenase in untreated control. b. Nephrotic. ($\times 10$).

FIG. 2. a. Appearance of renal cytochrome oxidase in untreated control. b. Nephrotic. ($\times 10$).

mitochondrial fractions of renal tubular epithelium of rats subjected to intraperitoneal injections of ovalbumin. Droplet formation, conspicuous in the tubular epithelium of rats with aminonucleoside nephrosis, has been considered as morphologic evidence of increased tubular reabsorption of protein.

Comparison of the results obtained manometrically and histochemically indicates that the latter technic is capable of detecting moderate losses of respiratory activity within the renal tubules and is not dependent upon an all or none phenomenon. In this regard our findings are similar to those of Cascarano and Zweifach(9) who found the oxygen consumption of fresh kidney slices under varying conditions to parallel endogenous values for 2, 3, 5 triphenyl tetrazolium chloride reduction.

Summary. A moderate decrease of QO_2

and a reduction in the intensity of the histochemical stains for succinic dehydrogenase and cytochrome oxidase have been observed in kidneys from rats with nephrosis induced by aminonucleoside. The possible relationship of these alterations to proteinuria is discussed.

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Antibody Production in Mice Exposed Intermittently to Radium Gamma Rays. (23772)

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Recovery processes taking place between successive irradiations are thought to account for the increased LD₅₀ and prolonged survival time reported for mice(1,2) and for rats(3) when the radiation was administered in 2 or more exposures made at varying time intervals. Mortality and survival times following radiation exposures in the lethal range and given as divided doses have been considered theoretically to be measurements of the summed injury to several physiological processes(4). Some of these processes, such as hematopoiesis, blood coagulation, defense against infection and antibody production following acute exposures to ionizing radiations have been studied in great detail. Less attention has been given to the study of injury to any of these processes following intermittent irradiation. Observations on the immune response following divided-dose exposures to ionizing radiation are of interest because measurement of injury to this physiological process may be made following sublethal exposures with good accuracy by procedures now available. Furthermore, studies of antibody production following intermittent exposure to radiation provide some information on injury and recovery processes involving the cellular mechanisms of protein synthesis. In the experiments here described antibody production and total circulating leucocyte counts were studied in mice following their intermittent exposure to radium gamma rays.

Materials and methods. Male NIH mice used throughout these experiments were 5 to 8 weeks of age at the beginning of the radiation exposure. Litters were represented in each of the treatment groups and mice exposed to radiation were confined in wood

boxes containing 5 to 8 animals each. Control animals were housed in steel cages in the animal rooms and at the end of the exposure both the irradiated and control mice were transferred to individual cages. Mice of parallel groups were bled from the tail for circulating leucocyte counts on the second day after the end of exposure. Immunization was done on the third day and the mice were sacrificed for serums on the 8th day after the end of the irradiation. Mice of another experiment were given acute exposure to X-rays 4 days after the end of the "chronic" accumulation of 450 r from the radium source described below in order to see whether the prior exposures would be additive to the acute irradiation in their effect on antibody production. Immunization was done one week and leucocyte counts 6 days after the acute exposure. Serological procedures followed those previously described(5) except that the serums were frozen and stored at -20°C until titration within 3 to 4 weeks after sacrifice of the mice. Briefly, the serum hemolysin content was estimated after a single intravenous inoculation of a 2.5% suspension of washed sheep erythrocytes. Approximately 4.85×10^6 erythrocytes were given per gram of body weight. Serums were titrated by the procedure described by Taliaferro(6) and the hemolysin titers are expressed as the negative log of the serum dilution providing 50% hemolysis in a standard suspension of sheep erythrocytes.

The arrangement for exposing the mice to the radium source was described in detail by Lorenz(7) and provided 8.8 r or 2.2 r per 8 hour day. In the experiments described in this paper the total exposure dose to the mice

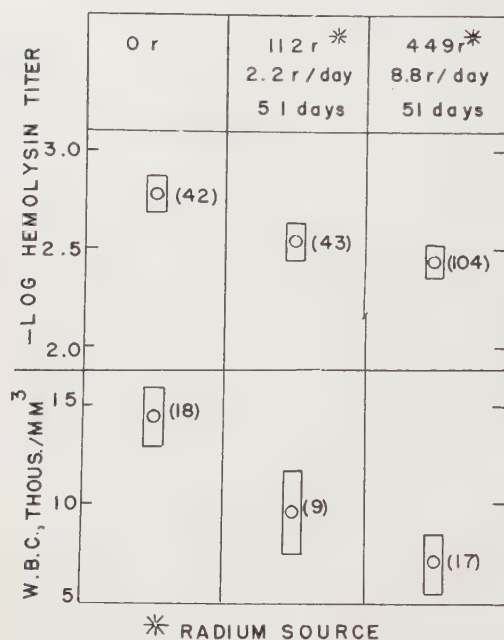


FIG. 1. Avg hemolysin titers on 5th day after immunization, above, and total circulating leucocyte counts, below, in NIH mice exposed to radium gamma rays at rate of 2.2 r or 8.8 r/8 hr day for 51 days. Immunization was done 3 days after end of radium exposure. Numbers in parentheses indicate numbers of animals/group and vertical bars represent 95% confidence limits of the means.

was about 112 r to one group while to the others about 450 r and these exposures were accumulated over a period of 51 days. The results of film dosimeter measurements* done on one occasion were in reasonable agreement with the more frequent ionization chamber measurements of the exposures. Acute exposures were made with a therapy X-ray unit operated at 200 Kvp and 20 ma with 0.51 mm Al and 0.25 mm Cu additional filtration and HVL = 0.76 mm Cu. The radiation was measured in air at 50 cm target distance to the center of the body.

Results. Average hemolysin titer (Fig. 1) in serums of mice of 2 experiments ($n = 47$ and 57) exposed to 8.8 r per 8 hour day for 51 days was -2.445 compared to -2.787 for their age controls ($n = 20$ and 22). The difference between control and experimental pooled means is significant at $p = < 0.001$

* The authors are grateful to Mr. Walter Cool of the Plant Safety Branch, Nat. Inst. Health, for conducting the film measurements.

while for the individual experiments the differences were significant at $p = < 0.01$ and < 0.05 respectively. It is of interest to note also in Fig. 1 that exposure of the mice to a total dose of 112 r resulted in a significantly lowered average hemolysin titer compared to the controls.

Average circulating leucocyte counts for parallel groups of mice simultaneously exposed to the radium dropped from 9.6×10^3 cells/mm³ in the 2.2 r/day group to 7.1×10^3 cells/mm³ in mice exposed to 8.8 r/day as compared to 14.4×10^3 cells/mm³ for control mice as shown in the Figure. Mean leucocyte counts for the treated mice are significantly below, $p = < 0.05$, the control mean. Comparison of the data presented in Fig. 1 shows that there is a tendency for the highest radiation dose to be associated with the lowest mean values both for the hemolysin titers and total leucocyte counts.

Impairment of the antibody producing capacity of the mice resulting from the radium gamma ray exposures in the experiments just described was small though highly significant. Table I summarizes the results of an experiment to test whether the exposure of mice to a dose of 450 r accumulated over a period of 51 days at the rate of 8.8 r per 8 hour day would have an additive effect if followed by an acute X-ray exposure. Four days after their exposure to 450 r in the radium field

TABLE I. Hemolysin Titer and Total Leucocyte Counts in NIH Mice after an Accumulated Exposure to 450 r Radium Gamma Rays followed by an Acute X-ray Exposure.

| Treatment | | Avg hemolysin titer \pm S.E.* | Avg total WBC $\times 10^3 \pm$ S.E.† |
|-----------|-------|---------------------------------|---------------------------------------|
| Radium | X-ray | | |
| 0 | 0 | $-2.83 \pm .08$ | 10.67 ± 1.36 |
| 450 r | 0 | $-2.72 \pm .13$ | $9.15 \pm .84$ |
| 0 | 150 r | $-2.34 \pm .08$ | $3.73 \pm .40$ |
| 450 r | " | $-2.26 \pm .09$ | $3.18 \pm .60$ |
| 0 | 250 r | $-1.70 \pm .14$ | $2.15 \pm .32$ |
| 450 r | " | $-1.41 \pm .17$ | $2.04 \pm .34$ |
| 0 | 350 r | < -1.0 | $1.33 \pm .23$ |
| 450 r | " | " | $.86 \pm .08$ |

* Mice, 18-20/group, were immunized 7 days after indicated acute exposure and serum samples obtained on 5th day after immunization.

† Leucocyte (WBC) counts were made on 9th day after end of exposure to radium, 9-10 mice/group.

groups of 18 to 20 mice were exposed to 150 r, 250 r or 350 r of X-rays (Table I). Average hemolysin titers for the mice exposed to the X-rays alone are only slightly higher than for the mice given a preliminary exposure of 450 r radium gamma rays. Mean titer for the 20 control mice was only slightly higher than the average titer for the mice receiving the radium exposure alone. It should be noted that the mice of this experiment were immunized 11 days later than those of the experiments described earlier in this paper. Total circulating leucocyte counts decreased as the acute exposures increased and in the mice given a preliminary exposure to radium the average counts were somewhat lower than in the mice given acute exposures only.

Discussion. It must be assumed from the results shown in Fig. 1 that recovery of the immune mechanism in the mice did not take place during the 16-hour interval which elapsed between successive exposures to as little as 2.2 r per 8 hour day. However, any residual injury to either antibody or leucocyte production which may have remained following the "chronic" exposure to 450 r of radium gamma rays was insufficient to alter significantly the response of the mice to a subsequent acute exposure to X-rays.

Unrecovered injury to the production of leucocytes following the radium exposure was also detectable from the lowered average counts observed and the values are in agreement with the leucocyte counts in LAF₁ mice following similar exposures(7). These results differ from the results of an acute X-ray exposure to partially shielded mice described previously(5) in which the total circulating leucocyte count was decreased without a reduction in the antibody producing capacity of the mice. In other experiments(8) antibody production recovered 3 to 4 weeks later than recovery of the leucocyte count in mice given

an acute exposure to 450 r of X-rays. In mice, therefore, hematopoiesis and antibody production do not appear to be closely dependent processes during the recovery from exposure to ionizing radiation.

Summary. Exposure of groups of mice to a radium source providing 8.8 r or 2.2 r per 8 hour day for 51 days resulted in significantly lowered antibody titers. In these experiments the mice were immunized on the third day after the end of the radiation exposure and the serums were sampled on the 5th day after immunization. Antibody titers and leucocyte counts following a single exposure of the mice to an acute X-ray dose 4 days after the end of an intermittent exposure to 450 r of radium gamma rays provided no evidence that the prior exposure produced an altered radiation sensitivity to either the hematopoietic process or the process of hemolysin production. The possibility that antibody synthesis in response to sheep erythrocyte antigen and leucocyte production are independent processes during the recovery from irradiation is discussed.

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Proliferation of Human Amnion Cells (FL Strain) in Submerged Culture.*† (23773)

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Ability of certain mammalian cells to proliferate in agitated fluid suspension (submerged culture) has been demonstrated utilizing a variety of equipment, nutritional media, and conditions(1-6). In our laboratories, strain HeLa (Gey) and L (Earle) have been cultured as discrete units in submerged culture in the spinner vessel, 25-500 ml(7); the New Brunswick fermentor, 1½-3 liters(8); and a 5 gallon stainless steel, water-jacketed, impeller agitated fermentor(9). The amnion cell as isolated from the human amniotic membrane has proved useful for isolation of certain viral agents(10). However, difficulties in obtaining a constant supply of satisfactory membranes are frequently encountered. Accordingly the establishment of a stable amnion cell line by Dr. Jorgen Fogh(11) presented the viral diagnostic laboratory with a procedure for procuring a uniform supply of this cell line. The present investigation presents data further simplifying the procedure demonstrating the ease of propagation of these cells in submerged culture thus making available at 2 or 3-day intervals fluid suspensions of these cells (1-1½ liters containing 1-1,500,000 cells/ml).

Methods. The strain of cells was supplied through the courtesy of Dr. Jorgen Fogh. The medium in our experiments consisted of 10% horse serum (heat inactivated at 56°C for 30 minutes) and 90% modified Eagle's medium.§ The equipment used consisted of the magnetic rotated Spinner system(7) and the standard impeller-agitated New Brunswick Fermentor (NBF)(8). Aeration of the culture was carried out by overlaying the fluid

with compressed air which had previously been water-saturated in order to reduce rate of evaporation of the culture medium. The volume of fluid for suspension and growth of the cells was varied from 100 ml (Spinner) to as much as 3 liters (NBF). Agitation of the cultures was carried out at an impeller rate of 60-250 rpm depending on the volume. It has been observed that the smaller volumes do not demand the high rate of agitation that is required for the larger volumes. The cells in these experiments were initially cultured on the surface of Povitsky bottles in a medium consisting of 10% horse serum (heat inactivated) and 90% Eagle's medium. Appropriate antibiotics were incorporated. 0.25% trypsin was used to remove the cells from the glass surface, prior to establishing the submerged cultures. Trypsinization was allowed to take place during constant shaking and for a time (5 min. or less) sufficient to remove the cells from the glass surface. The cells were then washed twice with Eagle's medium and placed in the submerged culture medium. It has been demonstrated by Westfall *et al.*(12) that changes in concentrations of glutamine, isoleucine, leucine, methionine and arginine

| L-amino acids (g/l) | Vitamins | (mg/l) |
|---------------------|------------------|--------------|
| Arginine HCl .021 | Biotin | 1 |
| Cystine .012 | Choline | 1 |
| Histidine HCl .008 | Folic acid | 1 |
| Isoleucine .026 | Nicotinamide | 1 |
| Leucine .026 | Pantothenic acid | 1 |
| Lysine HCl .026 | Pyridoxal | 1 |
| Methionine .008 | Thiamin | 1 |
| Phenylalanine .016 | Riboflavin | .1 |
| Threonine .024 | Glucose | 2.5 g/l |
| Tryptophane .004 | Phenol red | .01 " |
| Tyrosine .018 | Methocel (4000 | 1 " |
| Valine .024 | CPS Dow | |
| Glutamine .300 | Chem. Co. | |
| | methylcellulose) | |
| | Serum | 10% |
| | Penicillin | 100 units/ml |
| | Streptomycin | 50 µg/ml |
| | Mycostatin | 15 units/ml |

* This investigation was conducted under grant from the National Foundation for Infantile Paralysis.

† Contribution No. 21 from Microbiology in Medicine.

‡ These studies are in partial fulfillment of requirements for the MA degree.

| Salts | (g/l) |
|--------------------------------------|-------|
| NaCl | 7.0 |
| KCl | .4 |
| MgSO ₄ ·7H ₂ O | .2 |
| Na ₂ HPO ₄ | 1.44 |

occurred following growth of HeLa cells in a medium containing known amounts of these substances. Accordingly, it seemed reasonable to consider these substrates as possible limiting factors in continual cell growth. In our laboratories, Thomas *et al.*(13) have demonstrated that periodic additions of one of these substrates, arginine, circumvents the necessity for frequent medium changes during the cultivation of L cells over an extended period of time. Therefore, on initiation of the Spinner cultures, during medium changes or upon addition of new medium, glutamine, leucine, isoleucine, arginine and methionine were added at concentrations of 2 to 10 times that normally present in Eagle's medium. Even at the higher concentrations no demonstrable toxicity was observed. Puck *et al.*(14) have demonstrated that 0.4 $\mu\text{g}/\text{ml}$ of inositol increased the plating efficiency of various cell lines. It has also been reported that the concentration of inositol in blood plasma is in the range of 0.4-0.8 $\mu\text{g}/\text{ml}$ (15). Accordingly, inositol was likewise added to the medium at a concentration of 0.4 $\mu\text{g}/\text{ml}$. Enumeration of the cell population was made by withdrawing 2-5 ml aliquots and making direct counts in a standard hemocytometer. Trypan blue

staining of removed cells(7) was performed each day to determine the percentage of viable cells and thus the general state of the culture.

Results. The strain FL of human amnion cells readily proliferated in submerged culture, Fig. 1. Although the initial cell concentration was only 166,000 cells per ml, this population proved sufficient for initiating submerged growth. The cell multiplication rate approached a logarithmic growth curve with generation times of 25 to 36 hours (Fig. 1). Continuous cultivation in the submerged state for periods up to 1-2 months was readily achieved. At the points indicated in Fig. 1, cells were harvested from the stock tank (NBF) and placed in small Spinners, roller tubes or Povitsky bottles. (The cells showed no tendency to stick to the walls of the spinner-type culture vessel.) Greater than 98% of the total cell population remained viable as determined by the trypan blue staining procedure. The cells harvested at the indicated times grew well on the glass surfaces of the roller tubes or bottles, forming a typical monolayer sheet of cells in two days. The rapidity of the formation of the monolayer was directly related to the numbers of cells planted. Inoculation of glass monolayer or fluid suspension cultures with type 4 adenovirus demonstrated that these cells, as propagated, retained their susceptibility to this virus.

Summary. A method is described for propagation of human amnion cells (Strain FL) in Spinner-type culture vessels of volumes up to 3 liter capacity. Under these conditions, it is possible to maintain a stock culture which can supply cells for growth in roller tubes and Povitsky bottles without the necessity of a trypsinization procedure. The elimination of this dispersing procedure saves considerable time in handling of the cells. These cells after being propagated in submerged culture retain their susceptibility to type 4 adenovirus.||

|| Personal communication

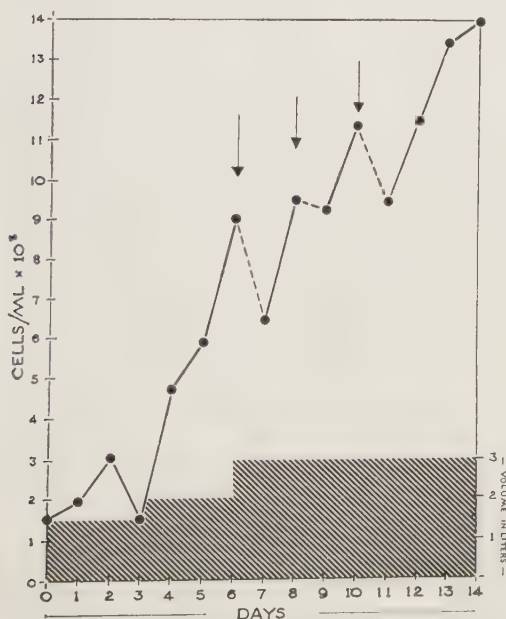


FIG. 1. Proliferation of F.L. strain in 3 l New Brunswick fermentor. Arrows indicate time of cell harvest or medium change.

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Comparative Susceptibility of Cell Cultures to Vaccinia Virus: Application to the Standardization of Smallpox Vaccine. (23774)

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Although the vaccine employed for immunization of man against smallpox is prepared from bovine or avian tissues, the assay method generally used for determining vaccine potency has employed still another species, the rabbit(1). Because considerable natural resistance and variable sensitivity are encountered in the routine performance of vaccine potency assays in rabbit epithelium, in recent years titration of vaccinia in the embryonated egg has supplanted and in some cases supplanted the rabbit dermal techniques(2,3). Whether based upon pock counts or lethal dose determinations the chorioallantoic method appears simpler, cheaper and more reliable than the rabbit assay(4a,b,c). The growth of human, simian, and bovine cells in tissue culture and the marked cytopathic effects produced by vaccinia virus in all mammalian cells studied offers still another means of testing smallpox vaccine and the attractive possibility that this biologic could be simultaneously assayed in cell systems homologous to the source and to the primate recipient. In addition plaque techniques may permit the use of more quantitative procedures in the manufacture and standardization of this biologic.

The following report is concerned with the susceptibility of several tissue cultures to vaccinia virus derived from bovine embryonic cells, calf lymph or embryonated eggs. The high susceptibility of monkey kidney monolayers to vaccinia virus has been utilized for comparative titrations of this agent by roller tube and plaque methods.

Materials and methods. Vaccinia seed virus was obtained as the National Institutes of Health Calf Lymph Reference Standard, Lot 1b, a lyophilized preparation of dermal pulp. Serial passages of this strain were initiated in various cell lines by inoculating 2 or more cultures of each type. When all the cells in the culture showed cytopathic changes, the supernatant fluids from one type were pooled and 0.1 ml of the pool passed to fresh tubes of the same tissue culture. The remaining pooled fluids from each passage were stored at -20°C until used. Certain of the calf lymph and egg vaccines were generously supplied by Cutter Laboratories, Lederle Laboratories, Merck, Sharp and Dohme, National Drug Co., Parke, Davis and Co., Wyeth Laboratories, Inc., and the New York City Board of Health. *Titrations* in rabbits (weighing 3-4 kg) were performed in two

TABLE I. Multiplication of Vaccinia in Bovine and Simian Cell Cultures.

| Source | Test cell | TCID ₅₀ %/ml at hours | | | | |
|-------------|---------------|----------------------------------|-----|-----|-----|-----|
| | | 30 | 48 | 68 | 90 | 115 |
| Bovine lung | Bovine kidney | 4.5 | 5.5 | 5.5 | 4.0 | 3.5 |
| | Monkey " | 4.5 | 4.5 | 6.5 | 4.5 | 7.5 |
| " muscle | Bovine muscle | 4.5 | 6.5 | 7.5 | 6.5 | |
| | Monkey kidney | 6.0 | 6.5 | 7.5 | 6.5 | 7.0 |
| " kidney | Bovine " | 4.5 | 5.5 | 6.5 | 5.5 | 3.5 |
| | Monkey " | 6.0 | 7.5 | 6.5 | 7.5 | 6.0 |
| Monkey " | " " | 4.5 | 5.5 | 5.0 | 5.5 | |

fashions; by injecting 0.1 ml of the virus dilutions intradermally and observing for pock formation and by scarification (0.2 ml per scarified area) performed in accordance with the "Minimum Requirements: Smallpox Vaccines"(1) of the National Institutes of Health. Egg titrations were made by pock count on the chorioallantoic membrane of chick embryos as described by Jackson *et al.* (4b). Monkey and rabbit kidney epithelium cultures were prepared from the trypsinized organs by the method of Youngner(5), HeLa cell cultures as described by Scherer *et al.*(6). Serial passage bovine embryonic lung, muscle and kidney cultures were established from a single embryo as reported earlier(7).^{*} Roller tube cultures were used for serial passages and titrations whereas for production of large pools of virus 32 oz. prescription bottles containing 50 ml of medium were employed. Plaque cultures of monkey kidney epithelium used in certain experiments were prepared by a method similar to that of Dulbecco and Vogt(8). Prior to use all cultures were drained of spent medium and washed once with Hanks' salt solution. Bovine and monkey cell cultures were fed Eagle's basal medium(9) containing 0.5% bovine albumin as a protein source. HeLa cells were cultivated in Eagle's medium with 10% horse serum and rabbit kidney cells were grown in either Eagle's medium or 0.5% lactalbumin hydrolysate in Earle's salt solution(10) plus 10% horse serum. Monkey kidney monolayers for plaque titrations were fed Eagle's basal medium (2X concentrated) supplemented with 20% skim milk; a medium sug-

gested by Dr. S. Baron. Virus titrations were performed by inoculating 3-4 tissue cultures per dilution with 0.1 ml of virus diluted in Hanks' salt solution or in the case of plaque counts 0.5 ml of virus into triplicate bottles. Tubes were then fed with 0.9 ml of medium and plaque bottles overlaid with 5.0 ml of 3% Noble's agar plus the medium described above. In experiments in which titers were compared by roller tube and plaque methods (Table IV) the same medium was employed, *i.e.*, Eagle's basal medium with 10% skim milk. A tube was read as positive when at least one area of vaccinal necrosis had developed. In the case of plaque preparations the time of virus contact before adding overlay was found to be of importance. Accordingly a 6-hour interval at 37°C was adopted as standard procedure between inoculation of virus and addition of overlay.

Results. Growth of vaccinia in serial passage bovine embryonic tissue. Calf lymph vaccinia was readily propagated in bovine cell cultures derived from lung, kidney or muscle tissue, in HeLa cells and monkey and rabbit kidney epithelium cultures. In view of Wesslen's demonstration of bovine embryonic skin as a satisfactory source of smallpox vaccine(11), the behavior of vaccinia in bovine cell embryonic cultures was studied in some detail.

Growth curve experiments in bovine lung, muscle, kidney and, for comparison, monkey tissue cultures were performed with calf lymph virus which had a single passage in bovine lung, muscle or kidney. Culture fluids were sampled 30, 48, 68, 90 and 115 hours after infection and titrated in the homologous cell and monkey kidney cultures. The results of this experiment are shown in Table I. It

^{*} A portion of the bovine cell cultures was obtained from Microbiological Assoc., Bethesda, Md.

TABLE II. Comparative Cell Susceptibility to Vaccinia Grown in Bovine and Simian Tissue Culture.

| Source | Passage | TCD ₅₀ /ml in | | | | | Titer in rabbit | | |
|---------------|---------|--------------------------|---------------|---------------|------|---------------|-----------------|-------|------|
| | | Bovine Muscle | Bovine Kidney | Monkey kidney | HeLa | Rabbit kidney | Scarif.* | I.D.† | CAM† |
| Bovine muscle | 13 | 5.5 | 5.5 | 5.7 | 5.5 | 5.7 | 1: 1,000 | 4 | 5.8 |
| | 25 | 5.0 | 4.7 | 5.5 | 5.6 | 6.0 | 1: 100 | <4 | 4.3 |
| " kidney | 13 | 4.5 | 4.0 | 4.5 | 4.5 | 3.7 | 1:30,000 | " | 4.0 |
| | 25 | 4.0 | 4.5 | 5.0 | 4.3 | 4.3 | 1: 100 | " | 2.8 |
| " lung | 10 | 5.5 | 5.0 | 5.7 | | 5.5 | | 5 | 5.8 |
| | 25 | 5.6 | | 6.0 | | 6.5 | 1:30,000 | 5 | 6.0 |
| Monkey kidney | 13 | | | 6.0 | | | | | |
| | 25 | | | 5.5 | | | | | |

* 0.2 ml of virus dilution/scarified area.

† Per ml.

will be noted that the maximum yield in all tissues was reached approximately 3 days after infection. The yield of virus was comparable for each of the 4 cell lines, ranging from $10^{5.5}$ to $10^{7.5}$ TCD₅₀ per ml, and there was no difference in susceptibility between bovine or simian cells.

To obtain tissue culture strains of vaccinia, which might have developed a predilection for the host tissue type, calf lymph virus was further passaged in bovine muscle, kidney and lung and in monkey kidney tissue cultures. The 13th and 25th serial passages in bovine muscle and kidney, in monkey kidney and the 10th passage in bovine lung were then titrated in a variety of cells, the rabbit and CAM as shown in Table II.

Propagation in each of these tissues resulted in decreased but stable infectivity when compared with the original calf lymph which had a titer of $10^{7.5}$ in monkey kidney. The passaged strains titrated between $10^{3.7}$ and $10^{6.5}$ TCD₅₀ per ml in tissue cultures of bovine, simian or human cells and the chorio-allantoic membrane. In these systems no significant decrease occurred between 13 and 25 passages.

In contrast, the findings obtained by rabbit skin titration, using either scarification or intradermal injection, indicate a loss of infectivity for this host since the 25th bovine cell passage caused pocks at only a dilution of 1:100 and failed to elicit any intradermal reaction at 1:10,000. It is to be noted that on the basis of their infectivity for bovine or monkey kidney cells the concentration of virus in these 2 bovine lines had not changed between the 13th and 25th passage.

Comparative infectivity of smallpox vaccines in various systems. A clearer definition of gradation in cellular susceptibility to vaccinia virus was obtained when the infectivity of a group of commercial smallpox vaccines was determined using tissue cultures, rabbits, and embryonated eggs.

A total of 10 vaccines, 9 of calf and one of egg origin and from 7 commercial laboratories were tested. All of these had met the potency requirements of the National Institutes of Health. As can be seen from Table III three ranges of susceptibility were encountered. Tested on monkey kidney these biologics had infectivity titers in the region of 10^8 TCD₅₀ per ml with an average of 7.5. HeLa and rabbit kidney cells, the chorioallantoic membrane and bovine cells constituted a second order of susceptibility with, in certain instances, a 2 log difference in titer as compared with that in monkey kidney. It should be noted that in 4 instances different lots of calf lymph titrated $10^{8.0}$ or higher in the monkey kidney whereas these same products possessed 0.3 to 2.0 logs less virus on the basis of the rabbit intradermal or CAM titration. This greater susceptibility of monkey kidney tissue culture over that of bovine cells was also found during an experiment designed to determine the yield of vaccinia virus per cell after 27 passages in bovine muscle tissue culture. This strain of virus had a calculated yield of 2.1 infectious units produced per cell when titration was made in bovine kidney as compared with a yield of 20 infectious units per cell when the same material was titrated in monkey kidney.

Titration in the rabbit skin by intradermal

TABLE III. Infective Titration of Smallpox Vaccines in Various Systems.

| Vaccine | Log TCD ₅₀ /ml | | | | | Rabbit scarif. titer† | ID ₅₀ /ml | |
|----------|---------------------------|------------------|------------------|------|------------------|--------------------------|----------------------|------------|
| | Bovine Muscle | Bovine Kidney | Monkey kidney | HeLa | Rabbit kidney | | Rabbit I.D. | Egg CAM |
| NIH Ref. | | 6.7 | 8.0 | 7.3 | 7.5 | 1:10,000 | 7.2 | 7.7 |
| 1* | 4.5 | 5.5 | 4.5 | | 4.5 | | | 4.5 |
| 1A | | 7.3 | >8.0 | 6.7 | 7.5 | 1:30,000 | 6.0 | 7.3 |
| 2 | 6.5 | 7.3 | 8.5 | | 7.0 | " | | 6.5 |
| 3 | 6.7 | 7.5 | >8.5 | | | " | | 6.5 |
| 3A | | 6.5 | | 7.0 | 7.5 | | | |
| 4 | 6.0 | 6.5 | >7.0 | | 7.0 | " | | 4.5 |
| 5 | | 7.0 | >8.0 | 7.2 | 7.5 | " | 7.0 | 7.2 |
| 6 | | 7.5 | 8.0 | 7.5 | 7.5 | " | 7.0 | 7.2 |
| 7 | 6.3 | | 7.3 | 7.0 | 7.7 | " | 7.5 | 7.3 |
| 8 | | 7.5 | 7.7 | 7.2 | 7.5 | | | |

* 1-7 are calf lymph vaccines; 8 is egg vaccine.

† 0.2 ml of virus dilution/scarified area.

inoculation seems comparable to the chorioallantoic membrane but less sensitive a procedure than when performed in monkey kidney cultures. The infectivity levels established by scarification of the rabbit were at the low end of the scale. In fact, Vaccines #2 and #4 gave only a single pock at a dilution of 1:30,000 on the scarified rabbit skin although their titers in a monkey kidney tissue culture were $10^{8.5}$ and greater than $10^{7.0}$ TCD₅₀ per ml respectively. Unfortunately there were no smallpox vaccines of substandard potency available with which to compare levels with those illustrated.

Relative sensitivity of monkey kidney tissue culture and the rabbit to vaccinia virus. When it became apparent that monkey kidney epithelium could be readily infected with vaccinia virus to high terminal dilutions a more detailed study was made of this culture involving a series of comparative titrations of calf lymph vaccine in plaques and roller tubes and in the scarified rabbit epithelium.

In agreement with Youngner's(5) earlier observations was the finding that successful plaque counts of vaccinia depended upon adequate attachment time of virus to cells and the medium used in the agar overlay. In a series of experiments with 25th passage monkey kidney-adapted virus the plaque titers were compared after varying periods of virus-cell contact at 37°C and with or without shaking during these intervals. Results of such an experiment, shown in Table VI, indicate that approximately 6 hours is required for complete adsorption of virus. Shaking for this

interval at a rate of 60 excursions per minute on a reciprocating type machine did not influence the infectivity end points. Nevertheless the practice of agitating the bottles once during the attachment period was adopted.

Comparison of the media in the overlay for the monkey kidney-vaccinia plaque system provided data which, although not susceptible to tabulation because most of the differences consisted of qualitative changes in the cell sheet rather than infective titer, suggested that the skim milk Eagle's medium formula was the most satisfactory. This was adopted as the standard medium. The overlay media tested were as follows:

- A. Medium 199
- B. Medium 199 with 20% skim milk
- C. Eagle's basal medium
- D. Eagle's basal medium with 20% skim milk
- E. Eagle's basal medium with 0.25% bovine albumin
- F. Youngner's medium(5)

Because the focal necrosis caused by vaccinia virus resembles somewhat the open areas which occur in monkey kidney monolayers microscopic examination of vaccinia plaques proved useful in recording endpoints. Characteristic rolled edges and loose swollen cells at the periphery of the necrotic plaque make differentiation of the specific virus lesion a simple matter. Agreement between roller tube and plaque titrations of vaccinia virus in monkey kidney epithelium is good. The results of one such experiment are shown in Table IV. Three lots of calf lymph virus were simultaneously titrated in cells of the same lot by

TABLE IV. Comparative Assay of Smallpox Vaccines in Monkey Kidney Tissue Culture Using Plaque and Roller Tube Methods.

| Vaccine | Cone. | Plaque | | Roller tube | |
|---------|------------------|--------|-------------------|-------------|---------------------------|
| | | Count* | Log titer PFU/ml | CPE | Log TCD ₅₀ /ml |
| 3A | 10 ⁻⁶ | TNC† | 10 ^{7.8} | 4/4 | 10 ^{7.6} |
| | 10 ⁻⁷ | 3 | | " | |
| | 10 ⁻⁸ | | | 1/4 | |
| 1A | 10 ⁻⁶ | TNC | 10 ^{8.0} | 4/4 | 10 ^{7.6} |
| | 10 ⁻⁷ | 5 | | " | |
| | 10 ⁻⁸ | | | 1/4 | |
| 5 | 10 ⁻⁶ | TNC | 10 ^{7.0} | 4/4 | 10 ^{7.5} |
| | 10 ⁻⁷ | .6 | | " | |
| | 10 ⁻⁸ | | | 0/4 | |

* Avg counts for triplicate cultures.
† Too numerous to count.

both methods using triplicate plaque bottles and quadruplicate tubes per dilution. The data indicate that a single plaque infectious unit resulted in a positive roller tube and endpoints were the same by either method. An-

other example of this is shown in Table V discussed below.

It has been long recognized that the titration of calf lymph vaccines on the scarified skin of the rabbit, a procedure based on Calmette and Guerin's work of 55 years ago(12), affords only a rough approximation of absolute infectivity. Although intradermal titration in rabbits, as suggested by Smadel *et al.* (13), is more consistent, the standard potency assay for smallpox vaccines in the U.S. continues to employ scarification. On the arbitrary assumption that titration in monkey kidney cultures yields an index more closely approximating the number of infective particles, it was considered profitable to conduct a series of titrations in tissue cultures and scarified rabbits with lots of fresh calf lymph and egg vaccine to determine the differential of sensitivity of the two methods. Both plaque and roller tube cultures were employed. The detailed results included in Table V illustrate the consistency of the pro-

TABLE V. Comparative Titration of Commercial Smallpox Vaccines in Monkey Kidney Tissue Culture and the Scarified Rabbit.

| Vaccine | Source | Exp. No. | Monkey kidney tissue culture | | Rabbit No. | Scarified rabbit* | | | | |
|----------------|-------------|----------|------------------------------|-----------------------|------------|-------------------|--------|--------|----------|----------|
| | | | Plaque PFU/ml | TCD ₅₀ /ml | | Pocks/dilution | | | | |
| | | | | | | Log | 1:1000 | 1:3000 | 1:10,000 | 1:30,000 |
| NIH control 1b | Calf lymph | 1 | 7.3 | 7.0 | 1-4 avg | Conf.† | Conf. | Conf. | 5 | |
| | | 2 | 7.7 | 8.0 | | | | | | |
| | | 3 | 7.8 | 7.8 | | | | | | |
| | | 4 | 7.6 | 7.6 | | | | | | |
| 1a | <i>Idem</i> | 1 | 8.0 | 7.6 | 1 | " | " | " | Conf. | |
| 3a | " | 1 | 7.8 | 7.6 | 1 | " | " | " | " | |
| 5 | " | 1 | 7.0 | 7.5 | 1 | " | " | " | 17 | |
| 9 | " | 1 | 7.6 | 8.0 | 1 | Not done | | | | |
| | | | | | | 2 | 14 | 14 | 3 | 1 |
| | | | | | | 3 | 7 | 5 | 1 | 0 |
| | | | | | | 4 | 15 | Conf. | 10 | 5 |
| | | 3 | 7.5 | 7.5 | 2 | 2 | 2 | 1 | 2 | |
| | | | | | | 1 | 3 | 5 | 3 | 1 |
| | | | | | | 2 | 11 | 9 | 7 | 3 |
| | | | | | | 3 | Conf. | Conf. | 8 | 4 |
| | | 2 | 7.7 | 8.3 | 4 | 8 | 5 | 5 | 3 | |
| | | | | | | Not done | | | | |
| 10 | " | 1 | 8.5 | 8.3 | 1 | Conf. | Conf. | Conf. | 2 | |
| | | | | | | 2 | " | " | " | Conf. |
| | | | | | | 3 | " | " | " | " |
| | | | | | | 4 | " | " | " | " |
| | | 3 | 8.6 | 8.3 | 1 | " | " | " | " | |
| | | | | | | 2 | " | " | " | 5 |
| | | | | | | 3 | " | " | " | Conf. |
| | | | | | | 4 | " | " | " | " |
| | | 1 | 8.0 | 8.0 | 4 | " | " | " | " | |
| | | | | | | Not done | | | | |
| 11 | Egg | 1 | 8.0 | 8.0 | | | | | | |

* 0.2 ml/site

* 0.2 ml/site. † Confluent lesions.

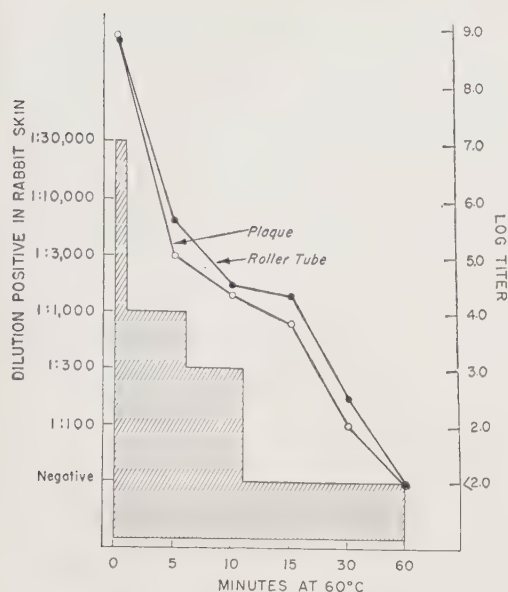


FIG. 1. Thermal inactivation of vaccinia virus as determined in monkey kidney tissue culture and scarified rabbit skin.

cedures as well as the inadequacy of the rabbit method in reflecting the infectiousness of a preparation. For example, Reference Vaccine 1b was retested at 4 intervals over a period of 6 months. By tissue culture this product had a titer of $10^{7.0}$ to $10^{8.0}$ TCD₅₀/ml with an average of $10^{7.6}$ TCD₅₀/ml and 4.3×10^7 PFU/ml. However an average of only 5 pocks (several rabbits were negative) was elicited when 0.2 ml of a $10^{-4.5}$ (1:30,000) concentration was tested on the scarified rabbit skin. Still more striking are the results of assays on Vaccines 9 and 10 which had monkey kidney infectious titers of $10^{7.9}$ and $10^{8.3}$ TCD₅₀/ml respectively. In 2 experiments involving 8 rabbits, Lot 9 produced poor lesions at all dilutions tested including 1:1000. Lot 10 was quite regular in its rabbit pathogenicity with an endpoint greater than 1:30,000.

To obtain additional data on the difference of titers measured by these two methods a thermal inactivation experiment was performed on the premise that partially inactivated vaccine would be titratable in monkey kidney cells after it had lost its rabbit infectivity. Such proved to be the case (Fig. 1).

Aliquots of a lot of calf lymph vaccinia

were submerged in sealed glass ampoules in a 60°C water bath for the incubation times shown. These were arbitrarily selected after preliminary experiments established a range for complete inactivation. The fractions were then simultaneously titrated in monkey kidney epithelium cultures and by rabbit scarification. When the infectivity of the lymph had decreased to approximately $10^{4.5}$ TCD₅₀/ml, the lowest concentration positive in rabbit skin was $10^{2.5}$. After 15 minutes at 60°C, there were approximately 4 logs of residual virus infectivity as measured in tissue culture but none detectable on rabbits at the highest concentration tested. Thus, although considerable loss of potency can be established by the rabbit scarification method it by no means can be used to determine residual infectivity of vaccinia virus in calf lymph.

Discussion. Highest yields of vaccinia virus were found in first or second bovine or simian tissue culture passages from calf lymph after which titers appeared to stabilize at a lower level. No evidence of species adaptation, as evidenced by enhanced infectivity for homologous or heterologous host cells, has been observed with serial passage of the virus. However, a significant qualitative change in its dermatropism for rabbit skin was noted even after 1 or 2 tissue culture passages. Whereas calf lymph vaccinia produced firm, well demarcated pocks on the scarified rabbit epithelium by the 5th or 6th day, the virus after passage in tissue culture caused a more accelerated but milder pock formation with less induration. Nevertheless, as demonstrated by Wesslen(11) vaccinia grown in bovine skin culture possessing titers of $10^{6.0}$ or greater is a satisfactory source of human smallpox vaccine even when pock formation in rabbits is of the less invasive type.

This observation is in accord with that of Buddingh and Randall(14) who noted a similar alteration of tropism without loss of infectivity in their studies of chick embryo smallpox vaccine. It is of interest that this modified dermatropism should occur even when calf lymph virus is serially propagated in cultures of calf embryonic tissue.

It should be stressed that although calf

TABLE VI. Relationship between Adsorption Time and Plaque Formation by Vaccinia Virus in Monkey Kidney Cells.

| Adsorption time (hr) | Exp. No. | Avg plaques/bottle* | | | |
|----------------------|----------|---------------------|------------------|------------------|------------------|
| | | 10 ⁻³ | 10 ⁻⁴ | 10 ⁻⁵ | 10 ⁻⁶ |
| 2 | 1 | TNC† | | 6 | |
| | 2 | | 1 | | 0 |
| 4 | 1 | " | | 1.5 | |
| | 2 | | 16 | | 0 |
| 6 | 1 | " | | 10 | |
| | 2 | | 24 | | 1 |
| 8 | 1 | " | | 3 | |
| | 2 | | 27 | | 0 |

* 0.5 ml virus dilution/bottle.

† Too numerous to count.

lymph vaccinia was most infectious for monkey kidney epithelium, it did not multiply to any greater level in this cell. We have no evidence that the practice of "lapinizing" or "humanizing" the calf lymph seed used for smallpox vaccine alters the direct infectivity of calf lymph for monkey kidney cells in tissue culture.

These experiments suggest that replicate titration in tissue culture provides a sensitive and reproducible technic for the measurement of the viral content of smallpox vaccines. A sample of the regularity of the data obtainable by this method is shown in Table IV which illustrates a typical vaccine titration. In simplicity it is comparable to the use of the embryonated egg and with plaque procedures, still greater exactitude may be attainable. Although the use of monkey kidney permits the assay in a cell species closer to that of the human recipient, it is premature to state whether such a tissue culture assay of smallpox vaccine accurately measures the quantitative and qualitative factors involved in successful human vaccination. At present it can only be said to constitute a titration system providing a close approximation of infectious units and one which can very read-

ily supplement the standard rabbit assay.

Summary. The infectivity of vaccinia virus in calf lymph for bovine, avian and simian cell cultures has been measured in tissue culture, embryonated eggs and rabbit epithelium. Monkey kidney roller tube or plaque titrations appear most sensitive to this virus and provide a simple and reproducible assay method which should have applications in the potency assay of smallpox vaccines.

We wish to thank Miss Elizabeth Jackson for performing the titrations on chorioallantoic membrane.

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Effects of Oxytocin and Blocking Agents upon Pituitary Lactogen Discharge in Lactating Rats.* (23775)

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It is known that nursing stimuli are necessary for maintenance of milk secretion and initiation of milk "let-down" by causing reflex discharge of lactogenic hormone from adenohypophysis and oxytocin from neurohypophysis(1). Because of the integration and coordination of these hormones in maintaining lactation it has been postulated that oxytocin might be the humoral link for release of lactogenic hormone(2,3). Basis for such postulation is mainly the ability of injected oxytocin to retard mammary gland involution in the rat. We thought it necessary to test the validity of this concept by determining if oxytocin could induce discharge of pituitary lactogen in nursing rats whose reflex release of lactogen and oxytocin have been blocked and to see if cholinergic and adrenergic components were involved in reflex discharge of lactogen as has been found previously in regard to release of oxytocin in response to nursing stimuli in lactating rats(4).

Materials and methods. Ninety-five adult primiparous lactating rats of the Sprague Dawley-Rolfsmeyer strain, weighing 270-320 g, were housed in individual cages and fed Purina Lab Chow and water *ad libitum*. Each litter was adjusted to 6 young shortly after birth and when 14 days old separated from their mother for 10 hours, then replaced and allowed to suck for 30 minutes. The mothers were treated as follows: Fifteen received single subcutaneous injections of 60 mg/kg Dibenamine-Cl⁺ 2 hours before nursing. Twenty-five others received single subcutaneous injections of 700 mg/kg atropine-SO₄ either 20 minutes or 2½ hours before nursing.

Remaining animals were anaesthetized with Nembutal (45 mg/kg *i.p.*) 10-20 minutes prior to end of isolation period. When completely anaesthetized each mother was laid on her side and her young replaced. Forty of these received oxytocin§ in doses of .05-.1, 3 or 6 USP/kg in a volume not exceeding .06 ml into the superior epigastric vein a few minutes after the young were replaced and while they were actively sucking. Fifteen similarly received distilled water. After nursing, each mother was killed, her pituitary gland removed rapidly, weighed and frozen until assayed for lactogenic hormone. Five glands were pooled for each assay. These were crushed in an agate mortar, suspended in a measured amount of distilled water and assayed in common adult pigeons by the Reece-Turner intradermal method(5).

Results. Data previously obtained(6) indicated a pituitary lactogen level of 2.44 Reece-Turner (R-T) units/100 g following isolation of mother and litter for 10 hours on 14th day postpartum (prenursing level). Following 30 minutes nursing, the level had dropped to 1.66 R-T units/100 g (post-nursing level). In the present investigation Dibenamine and Nembutal effectively blocked lactogen release from pituitary gland since nursing evoked no decline from control prenursing level (2.39 and 2.46 R-T units/100 g, respectively). Atropine injected 2½ hours before nursing also totally blocked lactogen release in response to nursing stimuli (2.35 R-T units/100 g) but only partial blockade occurred when the drug was administered 20 minutes prior to nursing (2.17 R-T units/100 g). Oxytocin injected *i.v.* into Nembutal-anaesthetized nursing rats in dose of .05-.1 USP/kg, which dose previously had been determined as the amount of hormone released as a result of nursing stimuli in 14-

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‡ Kindly supplied by Smith, Kline and French Laboratories, Philadelphia.

§ Kindly supplied by Armour Laboratories, Chicago.

TABLE I. Lactogen Content of 14-Day Postpartum Lactating Rat Pituitary Gland. Data obtained after 30 min. nursing following 10 hr isolation of mother and litter of 6 young.

| Treatment | Time of treatment prior to nursing (min.) | No. of rats | Avg wt of mothers (g) | Avg pituitary wt of mothers (mg) | Avg Reece-Turner lactogen | |
|--|---|-------------|-----------------------|----------------------------------|---------------------------|-------------|
| | | | | | Units/pituitary | Units/100 g |
| Control, prenursing* | — | 15 | 289.9 | 10.5 | 7.08 | 2.44 |
| Control, postnursing* | — | 15 | 292.5 | 9.3 | 4.85 | 1.66 |
| Dibenamine, 60 mg/kg subcut. | 120 | 15 | 293.7 | 9.9 | 7.03 | 2.39 |
| Atropine, 700 mg/kg subcut. | 150 | 15 | 289.2 | 10.8 | 6.79 | 2.35 |
| <i>Idem</i> | 20 | 10 | 277.1 | 9.6 | 6.00 | 2.17 |
| Nembutal, 45 mg/kg intrap. | 10-20 | 15 | 306.1 | 10.8 | 7.52 | 2.46 |
| <i>Idem</i> + .05-.1 USP/kg oxytocin intrav. | 10-20 | 10 | 289.7 | 10.6 | 7.22 | 2.49 |
| <i>Idem</i> + 3 USP/kg oxytocin intrav. | 10-20 | 15 | 312.0 | 11.3 | 6.88 | 2.22 |
| <i>Idem</i> + 6 USP/kg oxytocin intrav. | 10-20 | 15 | 286.8 | 11.4 | 6.13 | 2.14 |

* Data from Grosvenor and Turner(6).

day lactating rats(7), failed to alter pituitary lactogen from the control prenursing level (2.49 R-T units/100 g). Amounts of oxytocin 30x and 60x the upper physiological dose of .1 USP/kg produced but a slight diminution from control prenursing level (2.22 and 2.14 R-T units/100 g, respectively).

Discussion. We have shown(6) nursing stimuli evoke very rapid discharge of lactogenic hormone from the pituitary gland of lactating rats. In the present study, we have been able to prevent totally such discharge with Dibenamine and atropine injected in doses and at times these drugs previously have been shown to block oxytocin release in the lactating rat(4). The evidence in regard to blockade of oxytocin release was interpreted as indicating cholinergic and adrenergic links were involved somewhere in the neural pathway from mammary gland to pituitary gland. We believe, from results of the present study, a similar interpretation applies to lactogen discharge in response to nursing stimuli. Since it is well known such stimuli evoke reflex release of oxytocin and lactogen(1), it is possible there exists a common sensory pathway from mammary gland to hypothalamus. We suggest, therefore,

adrenergic and cholinergic components are involved, perhaps in the hypothalamic area, in simultaneous discharge of these hormones.

The role of oxytocin acting as a humoral agent responsible for lactogen discharge has not been substantiated by our present experiments. Nembutal has been shown(7) to block oxytocin release in response to nursing stimuli in lactating rats and, in the present investigation, lactogen discharge as well. Physiological doses of oxytocin failed to effect lactogen release in anaesthetized lactating rats though it may be argued only a small portion of the quantity administered i.v. actually reached the anterior pituitary gland. However, sufficient oxytocin certainly gained access to the pituitary when injected i.v. in doses 30 and 60 x the physiological level, yet insignificant discharge of lactogen occurred. If oxytocin were capable of evoking release of pituitary lactogen we should have obtained a decline from the prenursing level of 2.44 R-T units/100 g to a level approaching that resulting from 30 minutes nursing (1.66 R-T units/100 g). Meites (personal communication) and Meites and Turner(8) also have been unable to demonstrate any change in pituitary lactogen following administration of

oxytocin or vasopressin in the rat, guinea pig or rabbit.

The observations of Benson and Folley (2, 3) that oxytocin maintains involuting mammary gland of rats for a period of time in a state of active secretion following daily oxytocin administration must be explained in terms other than their suggestion that oxytocin causes discharge of lactogenic hormone from the anterior pituitary gland, attractive as it may appear.

Summary. 1) The effect of various compounds upon release of lactogenic hormone from the hypophysis in response to nursing stimuli has been studied in 14-day postpartum lactating rats. 2) Atropine, Dibenamine and Nembutal effectively blocked pituitary lactogen discharge since 30 minutes nursing evoked no decline (2.35, 2.39 and 2.46 Reece-Turner units/100 g, respectively) from previously obtained control prenursing level (2.44 R-T units/100 g). The normal level following 30 minutes nursing was previously determined as 1.66 R-T units/100 g. Since Dibenamine is a potent adrenergic blocking agent and atropine a cholinergic blocking agent, it is suggested cholinergic and adrenergic links are involved in the reflex arc responsible for lac-

togen discharge from rat pituitary gland. 3) Oxytocin injected i.v. into Nembutal-anaesthetized lactating rats in physiological doses failed to alter pituitary lactogen (2.49 R-T units/100 g) from the control prenursing level. Amounts of oxytocin 30 and 60 x this dose produced but a slight discharge. These results suggest oxytocin has no stimulatory effect upon pituitary lactogen release in the lactating rat, and are contrary to the recent hypothesis that oxytocin is a humoral link involved in lactogen discharge.

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Serotype Composition of the Adenovirus Group. (23776)

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A previous report (1) described 14 immunologically distinct viruses possessing the attributes of the adenovirus group (2). This communication describes 9 additional serotypes of human and monkey origin, as well as a subtype of type 7, designated 7a.

Materials and methods. Criteria for including a new virus in the adenovirus group have been described previously (1,3). All types reported here demonstrated characteristic cytopathogenicity for HeLa cell and monkey kidney tissue cultures, ether resistance, lack of pathogenicity for 1-day-old and adult mice and adult rabbits, and ability to demon-

strate complement fixing antibody responses in paired serums of at least 4 humans infected with other adenovirus types. The strains designated as prototypes represent, of necessity, the first strain of the type received and tested in this laboratory. Neutralization tests were performed in tube cultures of trypsin-dispersed rhesus monkey kidney cells,* maintained in medium 199. Adenovirus neutralization procedure 2 (3) was employed throughout, using the same criteria for neutralization with

* Received from Microbiological Associates, Bethesda, Md.

TABLE I. Adenovirus Types of Human Origin.

| Type | Prototype strain | Isolated by | Source of prototype strain | |
|-------------------------------------|------------------|----------------------|----------------------------|---|
| | | | Specimen | Type of case |
| <i>Previously reported types(1)</i> | | | | |
| 1 | Ad. 71 | NIH(3) | Adenoid | Hypertrophied tonsils & adenoids |
| 2 | " 6 | " | " | <i>Idem</i> |
| 3 | G.B. | " | Nasal washing | Common cold volunteer |
| 4 | RI-67 | Hilleman & Werner(4) | Throat " | Primary atypical pneumonia |
| 5 | Ad. 75 | NIH(3) | Adenoid | Hypertrophied tonsils & adenoids |
| 6 | Ton. 99 | " | Tonsil | <i>Idem</i> |
| 7 | Gomen | Berge(5) | Throat washing | Pharyngitis |
| 8 | Trim. | Jawetz(6) | Eye swab | Epidemic keratoconjunctivitis |
| 9 | Hicks | Kibrick(7) | Stool | Arthritis, rheumatoid?, myelitis? |
| 10 | J.J. | NIH(1) | Eye swab | Conjunctivitis |
| 11 | Slobitski | Kibrick(7) | Stool | Paralytic polio (Type I polio also recovered) |
| 18 | D.C. | NIH(1) | Anal swab | Niemann-Pick disease? |
| <i>Newly classified types</i> | | | | |
| 7a | S-1058 | NIH(Shelokov) | Throat swab | Undifferentiated respiratory infect. |
| 12 | Huie | Kibrick(7) | Stool | ? Non-paralytic polio |
| 13 | A.A. | NIH(Rosen) | Stool | Well child |
| 14 | DeWit | van der Veen(8) | Throat swab | ARD |
| 15 | Ch. 38 | Murray & Chang(9) | Eye swab | Conjunctivitis (early trachoma?) |
| 16 | " 79 | <i>Idem</i> | <i>Idem</i> | <i>Idem</i> |
| 17 | " 22 | " | " | " |

TABLE II. Adenovirus Types of Simian Origin.

| Type* | Prototype strain | Isolated by | Specimen |
|-------|-------------------|---------------------|----------------------------------|
| C-1 | Bertha | Sabin & NIH(1) | Chimpanzee stool |
| M-1 | S.V. ₁ | Hull(10) | Cynomolgus kidney tissue culture |
| -2 | CV2 | NIH | <i>Idem</i> |
| -3 | Abin. | " (Abinanti) | Air sample from monkey room |
| -4 | 2043 | (Rightsel & McLean) | Monkey kidney tissue culture |

* C = chimpanzee; M = monkey.

human and rabbit serums as were previously used in tests with HeLa cell cultures, with the exception that the definitive readings were made 24 and 48 hours, respectively, after the virus control cultures had demonstrated cytopathic changes greater than 1-plus.

Results. Tables I and II record the prototype adenovirus strains. In order to give type designations to the prototype viruses originating from species other than humans, while avoiding confusion with types of human origin, it is suggested that subgroups be established within the adenovirus group, *i.e.*, types of human, chimpanzee (C), and monkey (M) origin. This separation appears justified not only because of the source from which the strains were isolated, but also because of certain differences in adaptation to tissue cultures. Thus, all types of human origin, with the exception of type 18, the D.C.

strain(1), can be carried through serial passage in HeLa cell cultures without difficulty; when inoculated into rhesus monkey kidney cultures, they rapidly produce cytopathic effects, but generally cannot be carried for more than 1 or 2 additional passages without the use of concentrated inocula. Conversely, the types recovered from rhesus or cynomolgus monkeys propagate and pass well in rhesus kidney cultures, and produce rapid cytopathic changes on primary inoculation into HeLa cells, but cannot be carried in serial passage in HeLa cells. The chimpanzee type, however, passes in both monkey kidney and HeLa cell cultures.

The neutralization test results which establish each of the prototypes listed in Tables I and II as being immunologically distinct viruses, are summarized in Table III. Each of the 24 prototype rabbit antiserums was

TABLE III. Heterologous Neutralization Encountered in Classification of New Adenovirus Serotypes.

| Rabbit antiserum | Titer vs homologous virus (reciprocal) | Neutralization of heterologous prototypes* (titer in parentheses) |
|------------------|--|---|
| Type 1 | 640 | None |
| 2 | " | " |
| 3 | 2560 | " |
| 4 | 320 | " |
| 5 | 160 | " |
| 6 | 320 | " |
| 7 | " | 7a (1:5) |
| 7a | 640 | 7 (1:640); 11 (1:5); 14 (1:10) |
| 8 | >80 | None |
| 9 | 320 | " |
| 10 | 160 | " |
| 11 | >320 | 7a (1:5); 14 (1:10) |
| 12 | 320 | None |
| 13 | 80 | " |
| 14 | 320 | C-1 (1:10) |
| 15 | 160 | 4 (1:40); 9 (1:10) |
| 16 | >320 | 1 (1:5); 4 (1:40); 6 (1:5); C-1 (1:10) |
| 17 | 320 | None |
| 18 | " | " |
| C-1 | " | 14 (1:20) |
| M-1 | 80 | None |
| -2 | 1280 | " |
| -3 | 320 | 6 (1:20); 8 (1:5) |
| -4 | >1280 | None |

* All 14 previously reported prototype antisera tested at 1:20 or lower against all 10 new prototypes, and all new prototype sera tested similarly against all 23 heterologous types.

titrated against the homologous virus, and tested at a dilution of 1:5, 1:10, or 1:20 against each of the new prototypes; also, each of the rabbit antisera to the new prototypes was tested in a similar manner against each of the previously reported prototypes. Whenever heterologous neutralization was encountered, the test was repeated and the titer determined. For conciseness, Table III lists only the heterologous reactions obtained; all the other cross tests were negative at a serum dilution of 1:20 or lower. Comparable cross neutralization tests between the previously described prototypes have been reported previously (1) and are not recorded in the table. The most noteworthy cross reactions, other than those between 7 and 7a, which will be considered in detail below, were the reciprocal cross between type 14 and the chimpanzee type, and the low titer reciprocal cross between 7a and type 11. As observed previously, occasional low titer, non-reciprocal,

cross neutralization was also observed.

The 7a virus, while closely related to type 7, appears sufficiently distinct immunologically to justify its consideration as a separate adenovirus. Table IV shows results of representative cross neutralization tests with the type 7 prototype and various 7a strains, as well as representative typings of related strains. Despite variations in sensitivity of neutralization tests and different potency of rabbit antisera, the reciprocal tests clearly indicated that the viruses designated as 7a strains were consistently different from the prototype strain of type 7 (Gomen), and were not significantly different from one another. In a series of 16 reciprocal tests of 3 7a strains (S-1058, APC-H, and Ch. 334) against Gomen, the "R" values of Archetti and Horsfall (11)[†] were 0.09 to 0.35, with a median of 0.22, indicating a significant difference in antigenic composition. In contrast, comparison of the same 3 7a strains with each other gave "R" values in 4 tests of >0.50, 0.71, 0.97, and >1.0, indicating no significant difference among these strains. From the data in Table IV, it is seen that the apparent difference between Gomen and the 7a strains was not simply a result of variation between responses of different rabbits. Although the Gomen serum #1, which was pooled serum of 2 rabbits, discriminated more sharply between strains than the Gomen serum #2, both sera gave generally similar results. It is noteworthy that tests with the lower titer Gomen serum #1 gave the impression of a one-way cross reaction of Gomen with S-1058, whereas when the higher titer Gomen serum #2 was used, it was apparent that Gomen virus could stimulate formation of significant antibody to S-1058.

From Table IV, it is seen that neutralization tests with different strains of the same

$$\begin{aligned}
 \dagger R = & \sqrt{\frac{\text{titer of serum A vs virus B}}{\text{titer of serum A vs virus A}} \times \frac{\text{titer of serum B vs virus A}}{\text{titer of serum B vs virus B}}}
 \end{aligned}$$

all titers being determined in the same test.

TABLE IV. Representative Neutralization Tests with Type 7 and 7a Strains (4 Experiments). Figures represent reciprocal of neutralizing antibody titer of rabbit antiserum.

| Rabbit antiserum | Virus | | | | | | |
|------------------|--|--------------------------------|--|--|--|--|------------------------------------|
| | Type 7 strains | | 7a strains | | | | |
| | Gomen (prototype) Calif., 1954 (T.O.Berge) | Sekiya, Japan, 1956 (C.Tanaka) | S-1058, Wash., D.C., 1955 (A.Shelokov) | APC-H, Salisbury, Eng., 1955 (H.J.Pereira) | Ch-334, Saudi-Arabia, 1955 (R.S.Chang) | T-439, Sheffield, Eng., 1955 (D.A. J. Tyrrell) | GL2280, Ill., 1955 (J.T. Grayston) |
| G* serum #1 | 160 | | 5 | 40 | | | |
| S-1058 | 320 | | 320 | ≥ 1280 | | | |
| APC-H | 10 | | 20 | 80 | | | |
| G serum #1 | 80 | | | | 5 | | |
| S-1058 | | | 320 | | 640 | | |
| APC-H | | | | >80 | 20 | | |
| Ch-334 | 20 | | 20 | 320 | 20 | | |
| G serum #1 | ≥ 640 | | 10 | | | <10 | 80 |
| S-1058 | 640 | | 640 | | | 640 | 5120 |
| G serum #1 | 640 | 80 | 5 | | | | 40 |
| " #2 | 2560 | | 160 | | | | |
| S-1058 | 640 | 160 | 640 | | | | 2560 |

* G = Gomen.

type gave rabbit antiserum titers differing by as much as 8-fold. This difference probably resulted from the fact that the tests were not performed with a predetermined dose of virus based on endpoint titrations, but used a virus dilution selected to produce a 2-day incubation period. This conclusion is supported by the data in Table V, which records the results of an experiment in which type 2 rabbit antiserum was titrated against various dilutions of a type 2 virus suspension. With decreasing quantity of virus, the apparent serum titer increased more than 16-fold; it is striking that virus dilutions having the same incubation period, 2 days, gave antibody titers differing by 8-fold. The results of this experiment are roughly comparable to those obtained by Ginsberg(12) for neutralization of type 2 adenovirus in HeLa cell cultures, using entirely different criteria for evaluating neu-

tralization.

As a consequence of this variation in sensitivity of neutralization tests, differentiation of 7 and 7a strains can be made only by comparing the titers of the 7 and 7a prototype antisera when titrated against the virus in the same test (Table IV). However, for determining if a virus belongs to the 7-7a category, a test with a potent 7a antiserum alone would suffice. Preliminary evidence suggests that the antigenic differences detected with rabbit antisera are reflected to some extent in human antibody responses.

In view of the reciprocal low titer cross neutralization between type 14 and the chimpanzee strain (Table III), paired sera† of 3 persons infected with type 14 virus, all of which demonstrated neutralizing antibody rises to type 14, were tested in the neutralization test against the Bertha strain; one person demonstrated a rise from less than 1:4 to 1:16, and the other two responded from less than 1:4 to partial neutralization at 1:4. In view of the frequency of heterologous neutralizing antibody responses in persons infected with type 14(8), these results cannot be interpreted as indicating a closer relationship of type 14 to Bertha than to other adenovirus types, but do confirm the relationship of

TABLE V. Effect of Virus Dosage on Sensitivity of Neutralization Test. Adenovirus type 2 tested against type 2 rabbit antiserum.

| Virus dilution | Incubation period to CPE >1+ (days) | Rabbit antiserum titer (reciprocal) |
|----------------|-------------------------------------|-------------------------------------|
| 3: 1 | 1 | <<20 |
| 1: 1 | 1 | <20 |
| 1: 3 | 2 | 20 |
| 1: 10 | 2 | 80 |
| 1: 30 | 2 | 160 |
| 1:100 | 3 | 320 |

† Received through the courtesy of Dr. J. van der Veen.

adenoviruses of human and non-human origin. Further evidence of this relationship is the low level neutralization of types 6 and 8 by rabbit antiserum to the Abin. strain (Table III). Hull *et al.*(10) have recovered a number of viruses having the attributes of adenoviruses from monkey kidney tissue cultures; these agents, comprising Hull's cytopathogenic Group I, were tested against the prototype antisera of the monkey and chimpanzee types. S.V.₁₅ typed as M-4, and S.V.₂₃ as M-2. S.V.₁₁, S.V.₁₇, S.V.₂₀, S.V.₂₅, and S.V.₂₇ were not neutralized by any of the 5 serums. The OM86 strain, recovered from monkey stool by Cheever(13), typed as M-3.

Discussion. The multiplicity of adenovirus types, particularly the existence of closely related but distinct strains, such as 7 and 7a, raises the question of antigenic stability of the adenoviruses. Reciprocal tests of strains within types 1, 2, 4, and 6, widely separated in time and/or place, have shown no evidence of antigenic differences within these types. However, preliminary evidence suggests that there is antigenic variation within type 18 as well as type 7. It seems quite probable that adenovirus types differ in the rate at which serologic variants emerge.

From present knowledge, it does not appear that the distinction between type 7 and 7a is reflected in a difference in epidemiologic pattern. Both viruses have been recovered from military recruits, and one strain of type 7 and many strains of 7a have been encountered in the general population. The latter type 7 strain, from a case of epidemic keratoconjunctivitis, was isolated in Japan (personal communication), while 7a viruses have been isolated in the United States, Canada (14), England(15,16), France(17,18), and Saudi Arabia(9); the majority of 7a strains were from cases of pharyngoconjunctival fever, simple conjunctivitis, or febrile respiratory infections. The apparent tendency to specificity of human neutralizing antibody responses, if confirmed by more adequate testing, would be of importance in epidemiologic studies as well as in selection of proper strains for adenovirus vaccines. The differences in strains may be responsible for the variation in neutralizing antibody response to type 7

observed by Hilleman *et al.*(19).

Although it does not seem sufficiently important at present that strains of the type 7 group be routinely classified as to subtype, it is worthwhile that a standard nomenclature be employed when referring to typing of isolates. It is recommended that strains neutralized by a type 7 or 7a antiserum, but not further classified, be referred to as "viruses of the type 7 group," and that the terms "type 7" and "type 7a" be reserved for strains shown to be similar to the respective prototypes.

Summary. Seven additional adenovirus serotypes of human origin have been established, including a subtype of type 7, designated 7a. Also, 3 additional adenovirus types of simian origin are reported. It is suggested that the adenovirus group be divided into strains of human, chimpanzee, and monkey origin, with separate series of type numbers.

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Fluorinated Pyrimidines VI. Effects of 5-Fluorouridine and 5-Fluoro-2'-Deoxyuridine on Transplanted Tumors.*† (23777)

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Descriptions of the tumor-inhibitory properties of 5-fluorouracil and 5-fluoroorotic acid, members of the series of fluorinated pyrimidines developed in a collaborative research between the McArdle Memorial Laboratory of the University of Wisconsin and Hoffmann-La Roche Inc., have previously been reported (1-4). Earlier work at McArdle(5) had demonstrated that uracil-2-C14 was utilized for nucleic acid biosynthesis by Flexner-Jobling carcinoma to a greater extent than by most normal tissues. Accordingly, it appeared likely that an analog closely related to uracil might have a chemotherapeutic potential in the treatment of cancer. Because of the well-known alterations of biological activity produced by the substitution of fluorine for hydrogen in a number of classes of physiologically active compounds and because of the similarity in the Van der Waals radii of the fluorine and hydrogen atoms, a fluorinated uracil was desired. The fluorine atom was placed in the 5-position because the methyl group of thymine is attached to uracil at that locus, and it was anticipated that 5-fluorouracil would block thymine biosynthesis, as indeed is the case(1). As the result of the logical extension of studies of the fluorinated pyrimidine bases, the nucleosides, 5-fluorouridine and 5-fluoro-2'-deoxyuridine, were synthesized by Duschinsky *et al.*(6). This paper

reports on the tumor-inhibitory properties of these compounds; some of their biochemical and metabolic effects have been given elsewhere(1,7-9).

Methods. The fluorinated pyrimidines were prepared and supplied by Dr. Robert Duschinsky and his colleagues of Hoffmann-La Roche Inc.(3,6), and were injected intraperitoneally unless otherwise indicated, starting one day after transplantation. The treatment ordinarily involved daily injections for 7 days. Sarcoma 180 and Adenocarcinoma 755 were transplanted by trocar into groups of female Swiss and BDF₁ mice, and the volumes measured at various times after transplantation. The justification for volume measurements and the methods of calculation are presented elsewhere(2). The Ehrlich ascites carcinoma and L-1210 leukemia were carried in ascites form in female Swiss and BDF₁ mice respectively, and the survival times of the mice were recorded. A 5-fluorouracil-resistant line of the Ehrlich ascites carcinoma was also used(2). The Novikoff hepatoma was carried by intraperitoneal transplantation of minced tissue in female Holtzman rats and their survival times were observed.

Results. Toxicity. Some information on the toxicity of 5-fluorouracil has been reported elsewhere(2). At the present time toxicity studies on the nucleosides of 5-fluorouracil have been limited to experiments in albino mice (strain IS 32). Both compounds are characterized by comparatively low toxicities

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† Paper V in the series is reference 9.

when given in a single dose.

With 5-fluorouridine (FUR) the LD_{50} of a single dose was found to be 384 mg/kg by the subcutaneous route, 275 mg/kg by intraperitoneal, and 250 mg/kg by intravenous administration. Toxic doses produced loss of weight (up to 25%) and diarrhea; death occurred not earlier than 6 days and not later than 13 days after a single dose of FUR. Repeated subcutaneous injections required a considerable reduction of the dose. Six injections at 25 mg/kg/day were fatal; the mice succumbed between the 6th and 8th day with a loss of 34% of the starting weight. Experiments in mice implanted with tumors showed that FUR is tolerated only at doses of 4.5 to 5 mg/kg/day for periods of 7-10 days if given subcutaneously or intraperitoneally. 5-Fluoro-2'-deoxyuridine (FUDR) was less toxic. No acute lethal dose was reached by intravenous injection; 1000 mg/kg was tolerated by 3 of 5 mice, and 2 mice died on the 7th day with negligible weight loss. The only sign of toxicity was an extreme loss of hair, which occurred in all mice in the days following the injection and which persisted unchanged during the entire observation period of 14 days. A lethal single dose of FUDR was only attained by intraperitoneal injection; 1000 mg/kg was followed by diarrhea and emaciation and the mice died 6 to 8 days after the injection. A dose of 500 mg/kg was tolerated by 4 out of 5 mice, with one fatality which occurred on the 7th day. Significant weight loss has only been observed in one animal which lost 24% of its initial weight, but was still alive on the 14th day. The LD_{50} by intraperitoneal injection was, therefore, approximately 650 mg/kg. Subcutaneous injection of 1000 mg/kg resulted in a slight loss of body weight (average loss 14%) during the first 6 days. The weights then increased slowly. One out of 5 mice died on the 7th day, another on the 12th day. However, it is uncertain whether the deaths were caused by FUDR. Repeated subcutaneous injections of 100 mg/kg given once daily on 6 successive days were well tolerated; initial weight loss was negligible and was followed by nor-

mal gains. Up to 15 intraperitoneal doses of 50 mg/kg were found to be without toxic effects in mice implanted with tumors.

Microscopic examination of sections of the organs of mice having received fatal doses of FUR revealed as the most prominent pathological changes extensive hemorrhages in the lungs and severe hemorrhages in the sternal bone marrow. After administration of single doses (1000 mg/kg) of FUDR similar but less extensive changes were observed infrequently. One mouse which was sacrificed 3 days after the last of 6 subcutaneous doses of 100 mg/kg of FUDR was free of these lesions.

Sarcoma 180. The effects of the fluorinated pyrimidines on the growth of this tumor are shown in Fig. 1, where the survival, tumor volume in mm^3 , and weight change are plotted against days after transplantation. The abbreviations used are: 5-fluorouracil (FU), 5-fluorouridine (FUR), and 5-fluoro-2'-deoxyuridine (FUDR). The number of mice in each group are given in the parenthesis, and the dose is expressed as mg/kg/no. of days. It will be noted that in the case of 5-fluorouridine (FUR) a dose of 4.5 mg/kg/7 days caused a considerable weight loss, but no significant effect on the growth rate of the tumor. The deoxyriboside (FUDR) at a dose of 50 mg/kg/7 days when given by stomach tube (S.T.) was also ineffective. The small numbers of mice in the FUDR groups was necessitated by a limitation of supply of the compound. It will be observed that FUDR when given intraperitoneally at a dose of 50 mg/kg/7 days was more effective and produced less weight loss than an equimolar dose of FU (25 mg/kg/7 days). The numbers on the graph at each point represent the fraction of the tumor volume as compared to untreated controls. When FUDR was given by intraperitoneal injection at 50 mg/kg/14 days the tumors did not appear.

In a duplicate experiment, in which FUDR was given to a group of 6 mice by intraperitoneal injection of 50 mg/kg for a period of 15 days, no tumors developed during the treatment period and no growth was observed during 12 days following discontinuance of the drug injections. Three of the animals re-

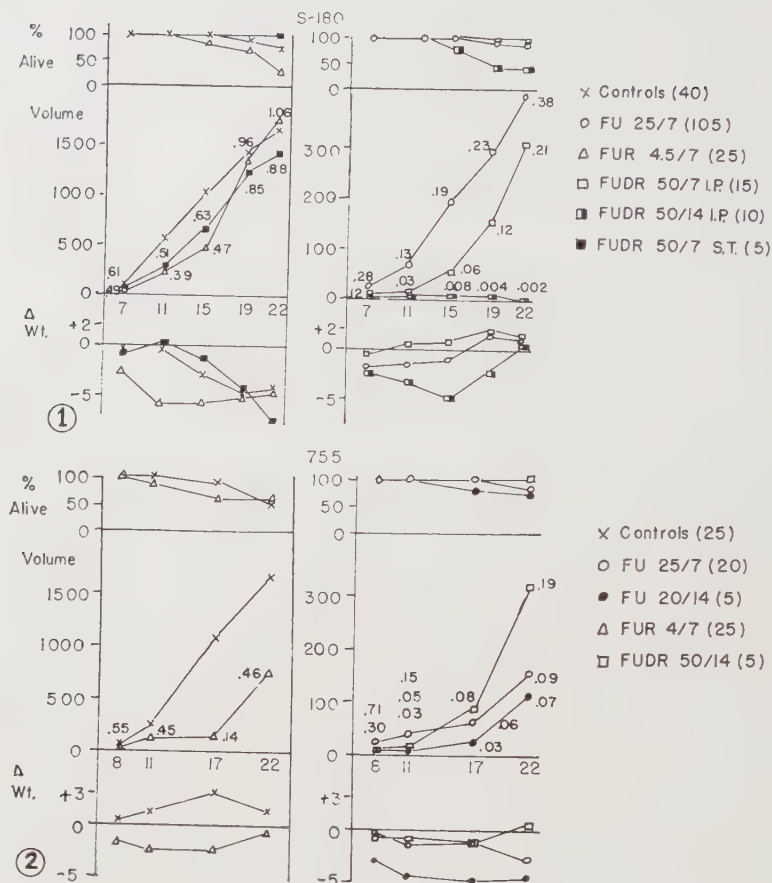


FIG. 1. Effect of fluorinated pyrimidines on growth of Sarcoma 180. Survival, tumor vol in mm³, and wt change in g plotted against days after transplantation. The dose is expressed as mg/kg/No. of days. Values in parenthesis are No. of mice in the experimental groups.

FIG. 2. Effect of fluorinated pyrimidines on growth of Adenocarcinoma 755. Survival, tumor vol in mm³, and wt change in g plotted against days after transplantation.

mained free of tumors to the 80th day when the experiment was terminated. The 3 remaining mice developed small tumors between the 28th and 41st day, and the tumors were removed for histological examination. The tissue structure of these specimens of retarded tumors resembled the microscopic appearance of Sarcoma 180. There were necrotic areas in the central parts of the tumors, but the peripheral zones showed all signs of active growth and mitotic activity. In one of the tumors removed on the 41st day enlarged cells were found, similar to those reported by Clarke *et al.*(10) in Sarcoma 180 tumors treated with 6-mercaptopurine. No such cells have been observed following treatment with 5-fluorouracil.

755 Mammary Adenocarcinoma. The effects of the drugs on this tumor are shown in Fig. 2. It will be noted that all exerted a considerable inhibition of tumor growth rate until the 17th day, but that the effect wore off by the 22nd day particularly with FUR and FUDR. It is clear that in this tumor 5-fluorouracil, when given at 25 mg/kg for 7 or 14 days is more effective than either fluorinated nucleoside.

L-1210 Leukemia. The per cent survival of mice bearing this tumor in ascites form is plotted against days after transplantation in Fig. 3. In this tumor, as in the 755, 5-fluorouracil is more effective than either of its nucleosides. A few mice treated with 5-fluorouridine survived longer than those treated

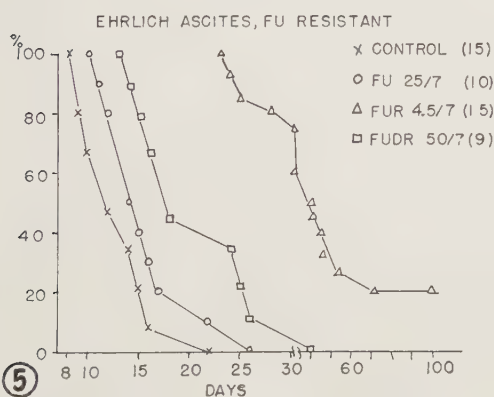
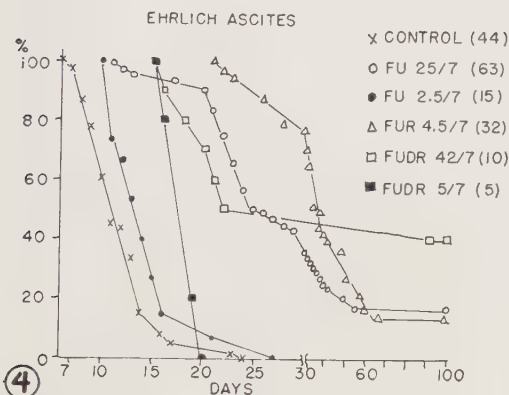
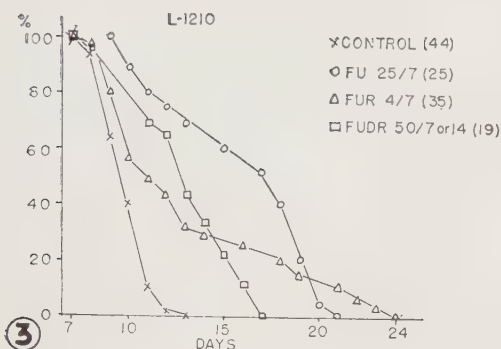


FIG. 3. Effect of fluorinated pyrimidines on survival of mice bearing L-1210 leukemia in ascites form. Per cent survival plotted against days after transplantation.

FIG. 4. Effect of fluorinated pyrimidines on survival of mice bearing Ehrlich ascites carcinoma. Per cent survival plotted against days after transplantation.

FIG. 5. Effect of fluorinated pyrimidines on survival of mice bearing a 5-fluorouracil-resistant line of Ehrlich ascites carcinoma. Per cent survival plotted against days after transplantation.

with 5-fluorouracil, but this is of questionable statistical significance. If the 50% survival

TABLE I. Novikoff Hepatoma.

| Days after transplantation on which rats died | | |
|---|------------------------------------|-------------------------------------|
| Controls | 5-Fluorouracil, 25 mg/kg/7 days | 5-Fluorouridine, 10 mg/kg/7 days |
| 8 | 11 | 21 |
| 8 | 12 | 22 |
| 9 | 12 | 30 |
| 9 | 13 | 100* |
| 9 | 13 | 100* |
| 10 | 15 | 100* |
| Avg 8.8 | 12.7 | |

* These rats were killed and found to be tumor-free at 100 days.

is noted, FU is considerably more effective than FUR.

Novikoff Hepatoma. The survival times of small groups of rats treated with FU and FUR are shown in Table I. Although a dose of 5 mg/kg/7 days of 5-fluorouridine produced considerable weight loss in mice, 10 mg/kg/7 days was well tolerated by rats, and at that dose the compound is a very effective inhibitor of this tumor. For comparison the lesser effect of 25 mg/kg/7 days of 5-fluorouracil is shown; this dose corresponds to 4 times the molar equivalent of FUR given in this experiment.

Ehrlich Ascites Carcinoma. The results of a series of survival time experiments are shown in Fig. 4. All 3 compounds were effective against this tumor only if given by intraperitoneal injection. It will be noted that 40% of the mice treated with 5-fluoro-2'-deoxyuridine survived for 100 days tumor-free whereas with 5-fluorouracil and 5-fluorouridine 18 and 15% respectively survived the experiment. Evidence that these mice were actually free of tumor cells was gained as follows. Sterile saline was injected intraperitoneally into 100-day survivors and withdrawn. Such lavage should be expected to pick up some tumor cells that may have been present. The washings were then injected intraperitoneally into recipient mice. None of these animals developed tumors over a 90-day period. Thus the treated animals were presumed to be cured of the tumor.

The comparison of these compounds at various doses is of interest. The most effective drug, as indicated by the number of surviving mice, is FUDR. This was given at

a dose of 42 mg/kg/7 days, which is approximately the equimolar dose of 25 mg/kg/7 days of FU. Under these conditions, FUDR is more effective than FU. 5-Fluorouridine, on the other hand, at a dose of 4.5 mg/kg/7 days is slightly more effective at prolonging the life of these mice than 25 mg/kg/7 days of 5-fluorouracil. The latter compound at 2.5 mg/kg/7 days, which is about equimolar to 4.5 mg/kg/7 days of FUR, is completely ineffective. The equivalent dose of FUDR, 5 mg/kg/7 days, has relatively little effect. Therefore we see that against the Ehrlich ascites tumor at optimal doses, 5-fluoro-2'-deoxyuridine is the most effective of the 3 drugs. However, 5-fluorouridine is by far the most efficacious on a mg/kg basis, since it is active at a much lower dose.

The results obtained with these 3 compounds in a 5-fluorouracil-resistant line of the Ehrlich ascites carcinoma(2) are shown in Fig. 5. Here the resistance to FU was almost complete, and there was a partial resistance to FUDR. On the other hand, FUR was just as active against the 5-fluorouracil-resistant line as the susceptible line, with 50% survival times of 40 days in each case.

Discussion. On the basis of biochemical knowledge it is reasonable to suppose that nucleosides of nucleic acid analogs might have altered biological and tumor-inhibitory properties. An example of this is the observation by Schindler and Welch that the riboside of 6-azauracil is active against cells of Sarcoma 180 grown in tissue culture, whereas 6-azauracil itself is not(11). On the other hand, Skipper *et al.*(12) found that 6-mercaptopurine and its riboside had approximately the same activities against Adenocarcinoma 755.

In the series of fluorinated pyrimidines it is of interest that in mice the toxicity by repeated doses of 5-fluorouridine is much greater than that of 5-fluorouracil, whereas the toxicity of 5-fluoro-2'-deoxyuridine is less than that of FU on an equimolar basis. The most striking conclusion which can be drawn from this work is the variation of response of these transplanted tumors to the 3 compounds. In Sarcoma 180 5-fluoro-2'-deoxyuridine is much more effective than 5-fluorouracil; 5-fluorouridine exerts no significant effect on

this tumor. In contrast, in both Adenocarcinoma 755 and L-1210 leukemia, 5-fluorouracil is the most effective of the 3 although FUDR and FUR exert significant inhibitions. In the Ehrlich ascites carcinoma, both nucleosides are more active than the free pyrimidine analog, with FUR effective at a much lower dose than the others. In the rat, 5-fluorouridine exerts less toxicity than in the mouse, and hence a dose of this compound twice as great as can be given to mice was much more effective than FU in inhibiting the growth of the Novikoff hepatoma. Insufficient supplies of FUDR did not permit tests against rat tumors. Whether the diversity in responses of the tumors to these 3 compounds is a reflection of different biochemical mechanisms of tumor-inhibition produced by these drugs, or whether it indicates differences in drug metabolism, tissue distribution, or cell permeability are matters currently under investigation.

The lack of cross-resistance between 5-fluorouridine and 5-fluorouracil in the FU-resistant line of the Ehrlich ascites carcinoma has obvious clinical implications, and the result is reminiscent of the observation by Handschumacher that a 6-azauracil-resistant line of *S. faecalis* was inhibited by azauracil riboside(13). In contrast, Skipper *et al.*(12) found that in 6-mercaptopurine-resistant strains of Adenocarcinoma 755 and *S. faecalis*, 6-mercaptopurine riboside had no effect. Whether this difference in response reflects a basic difference between the inhibitory mechanism of analogs of purine and pyrimidine nucleosides is not known, and studies of mechanism of action of the series of fluorinated pyrimidines in the resistant tumor are under investigation.

In spite of the inconsistent results obtained with various transplanted tumors, the undoubted tumor-inhibitory activities of 5-fluorouridine and 5-fluoro-2'-deoxyuridine make it appear highly desirable to overcome the severe technological difficulties of producing larger quantities of these substances in order to evaluate them clinically in human cancer.

Summary. 1. Activity of 5-fluorouridine (FUR) and 5-fluoro-2'-deoxyuridine (FU-

DR) against several transplanted tumors has been compared with that of 5-fluorouracil (FU). 2. In Adenocarcinoma 755 and L-1210 leukemia FU is more effective than either nucleoside, although the latter have significant tumor-inhibitory activities. 3. In Sarcoma 180, FUDR is much more effective than FU; FUR has no significant activity. 4. In the Ehrlich ascites carcinoma, both nucleosides are more effective in prolonging life than is 5-fluorouracil, with FUR demonstrating activity at a low dose. In a 5-fluorouracil-resistant line of the Ehrlich ascites carcinoma, 5-fluorouridine is as active as in the parent strain. 5. In mice, the toxicity by repeated doses of FUR is much greater than that of FU; FUDR is less toxic than FU. In rats, the toxicity of FUR is much less pronounced than in mice, and the compound was very effective in inhibiting the growth of the Novikoff hepatoma.

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Reversibility of Venular Dilatation and Congestion in Diabetic Subjects Over a Period of Hours. (23778)

JØRN DITZEL AND RAFAEL CAMERINI-DAVALOS (Introduced by Alexander Marble)

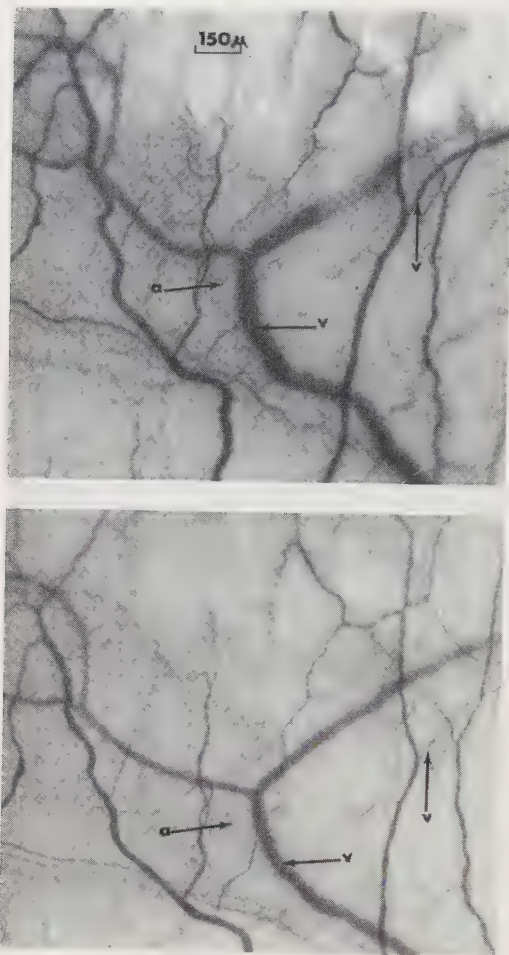
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The pathogenesis of the late diabetic sequelae, retinopathy and glomerulosclerosis, remains unknown. With ophthalmoscope (magnification 15 x) the initial visible change in the retina of diabetics consists of venous congestion (phleboopathy) and small sanguinolent dots (micro-aneurysms) (1,2). By using the stereoscopic microscope (magnification 100 x) on the bulbar conjunctiva, one observes in a great number of young diabetics a dilatation and congestion of the venules associated with exudation (3). A relationship has been found between the degree of retinal changes and the conjunctival alterations (4). Venular dilatation in the conjunctiva is apparently a reversible change which varies in

degree when observed at weekly intervals (5).

This investigation was undertaken to determine (1) whether venular dilatation fluctuates over a period of hours and (2) if so, whether it can be correlated with blood sugar levels or with time after administration of insulin.

Material and methods. Fifteen diabetic subjects admitted to the Hospital Teaching Clinic for regulation were chosen for study. Other than diabetes and its complications the subjects were free from disease. None had ketoacidosis. They included 6 females and 9 males whose ages varied from 10 to 43 years. The duration of diabetes ranged from 6



a, arteriole; v, venule

FIG. 1. Case R. C. Reversibility of venular dilatation and congestion occurring over period 8 a.m. (top) to 5 p.m. (bottom) of the same day.

months to 26 years. All took insulin, usually isophane (NPH) insulin alone or in combination with a small dose of crystalline insulin administered in the morning. The conjunctival vascular bed of the patients was microscopically studied and photographed from 2 to 5 times at irregular intervals between 8 a.m. and 6 p.m., using methods previously described (5,6). The vascular changes were evaluated and graded by measurements from the enlarged photomicrographs. If the calibers of identical vessel segments differed more than 100% at different times, the change was classified "marked." If the calibers differed less than 100%, the change was classified "moderate." In addition, blood glucose

determinations (Somogyi-Nelson method) (7) were obtained three times during the day of examination, usually before breakfast and in the late forenoon and late afternoon. Control conjunctival studies were simultaneously performed on 15 healthy non-diabetic hospital employees of comparable ages.

Results. Seven out of the 15 diabetics demonstrated caliber changes in the venules within a period of hours. In contrast *none* of the control subjects showed such vascular changes. Of the 7 diabetics, 3 showed marked changes (Fig. 1) while in the remaining 4 patients the alterations were classified as moderate. With regard to the underlying cause of the reversible venular congestion, the following observations might be of interest: (1) the caliber changes occurred only among the 10 diabetics in whom venular dilatation (Vascular Pattern Change I) (5) was present at the initial observation; (2) in most of the diabetics exhibiting changes during the day, the venular dilatation tended to disappear during the later afternoon corresponding to the time at which isophane (NPH) insulin has its maximum effect; (3) in the diabetics showing vascular alterations, the initial observations were made early in the morning and thus the presence of more pronounced venular dilatation corresponded to the time when the effect of the isophane (NPH) insulin administered the previous day was minimal.

No relationship was apparent between the venular response change and duration of diabetes, size of insulin dose, clinical hypoglycemia or actual blood sugar levels.

Conclusion. Preliminary observations demonstrate that the venular dilatation and congestion observable in the conjunctival vascular bed of diabetics may often fluctuate within a period of hours. Venular dilatation was, as a rule, least marked in the later afternoon, at the time when dietary intake and insulin effect may be expected to produce the optimal daily metabolic pattern.

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Incidence of Parthenogenetic Development in Eggs Laid by Three Strains of Dark Cornish Chickens. (23779)

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Cells of parthenogenetic origin were observed microscopically in the blastodiscs of newly laid infertile avian eggs by Oellacher (1); Duval(2); Lacaillon(3); Bartelmez and Riddle(4), as well as by others. This literature has been reviewed by Olsen and Marsden (5). More recently, Kosin(6) reported finding nucleated cells in 15% of the germinal discs of infertile, newly-laid White Leghorn and Barred Plymouth Rock eggs. Olsen and Marsden(7) examined macroscopically the germinal discs of infertile chicken eggs following a 7-9 day period of incubation. A limited development of the germinal disc was observed in 13.5% of Dark Cornish eggs and 1.5% of the Silver Cornish eggs. No parthenogenetic development could be detected by this method in infertile eggs laid by Rhode Island Red and New Hampshire hens.

Materials and methods. Infertile eggs from 3 strains of Dark Cornish chickens were examined both microscopically and macroscopically to determine the relative incidence of parthenogenetic development. Sixty-four virgin Dark Cornish pullets were used in the testing. 11 birds (Strain A) were F₁ progeny from matings of hens from a flock maintained at Agricultural Research Center with males from a commercial source. Strain B was composed of 12 birds from another commercial source and Strain C was composed of 41 birds from a third source. Whether the birds from the 3 commercial sources were related is not known but it is considered unlikely. The first 2 eggs laid by each of these hens were broken out when laid, and the blastodisc of each was fixed, sectioned, and stained with Heidenhain's Iron Hematoxylin. Each blastodisc

was studied microscopically and if either egg from each hen showed parthenogenetic development, the bird was classified as a producer of parthenogenetic eggs. This technic was similar to that used by Kosin(6). All eggs subsequently laid through a 5-month period were incubated 9-10 days and then broken-out and any macroscopically observable parthenogenetic development recorded. This technic was similar to that described by Olsen and Marsden(5). The results obtained at both times of observation (Table I) were recorded on an egg and bird basis, *i.e.* the number of eggs which showed parthenogenetic development in the total number of eggs laid, and the number of hens which laid one or more eggs showing parthenogenetic development in the total number of hens tested.

Results. All 11 birds of Strain A produced eggs showing parthenogenetic development at

TABLE I. Incidence of Parthenogenesis in 3 Strains of Dark Cornish Chickens Expressed as Percent of Birds and Percent of Eggs Observed Microscopically at Laying and Macroscopically after Incubation.

| Strain | At laying | | | After incubation | | |
|--------------------------------|-------------------|----------------------|-----|-------------------|----------------------|------|
| | Total No. obs. | Partheno- genetic | | Total No. obs. | Partheno- genetic | |
| | | No. | % | | No. | % |
| <i>Incidence on bird basis</i> | | | | | | |
| A | 11 | 11 | 100 | 11 | 7 | 63.6 |
| B | 12 | 10 | 83 | 12 | 4 | 33.3 |
| C | 41 | 23 | 56 | 41 | 3 | 7.3 |
| Total | 64 | 44 | 69 | 64 | 14 | 21.9 |
| <i>Incidence on egg basis</i> | | | | | | |
| A | 22 | 22 | 100 | 510 | 20 | 3.90 |
| B | 24 | 16 | 66 | 754 | 5 | .66 |
| C | 82 | 35 | 43 | 1314 | 5 | .38 |
| Total | 128 | 73 | 57 | 2478 | 30 | 1.16 |

lay, 83% (10 of 12) of Strain B birds, and 56% (23 of 41) Strain C birds had parthenogenetic development in newly laid eggs. Likewise 63.6% (7 of 11) Strain A birds, 33.3% (4 of 12) Strain B birds, and 7.3% (3 of 41) of Strain C birds produced eggs showing parthenogenetic development as observed after 9-10 days incubation. On an egg basis the microscopically observed incidence of parthenogenesis (57%) establishes the Dark Cornish breed as the highest producer of eggs showing parthenogenetic development of all domestic breeds of chickens reported on to date.

These data indicate definite strain differences within the Dark Cornish breed with regard to incidence of parthenogenesis. While Olsen(8) suggests that heredity is not exclusively in control, there is strong indication here that genetic factors are significant. Genetic differences are quite evident at the breed level since the incidence in these stocks is four times that reported by Kosin(6) in 2 different breeds of chickens.

It is apparent from the method employed that Olsen and Marsden(5) did not count those eggs which may have had parthenogenetic cells in the blastodisc at laying but did not develop further when incubated. It

would be interesting to know whether some of the eggs considered non-parthenogenetic by Olsen and Marsden would show parthenogenetic cells if examined at the time of lay.

Summary. Infertile eggs from 3 strains of virgin Dark Cornish pullets were examined either microscopically at laying or macroscopically after 9-10 days incubation to determine relative incidence of parthenogenetic development. The data indicate the incidence of parthenogenesis may vary not only in different breeds or strains of birds, but also the observed incidence may vary with different technics of observation and different times at which these observations are made.

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Some Sources of Error in the Akerfeldt Test for Serum Oxidative Activity.* (23780)

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The procedure recently introduced by Akerfeldt(1) for estimating serum oxidative activity is based on the rate at which color develops after equal parts of serum and of a 0.1% aqueous solution of N, N'-dimethyl-p-phenylenediamine (DPP • 2HCl) are mixed in the absence of added buffer. According to Akerfeldt, normal sera caused little color change in the first 3 to 5 minutes whereas sera from patients with psychoses (and with

many other diseases, as well as normal pregnancy) caused rapid development of color. However, observations made in these laboratories demonstrated that Akerfeldt's method does not accurately measure oxidative activity and cannot be used as a test for psychosis.

Observations. 1) *Temperature:* We found that small variations in initial temperature of the reaction mixture, or merely absorption by the mixture of heat generated in the spectrophotometer, significantly altered the

* Aided by grant from Donner Foundation.

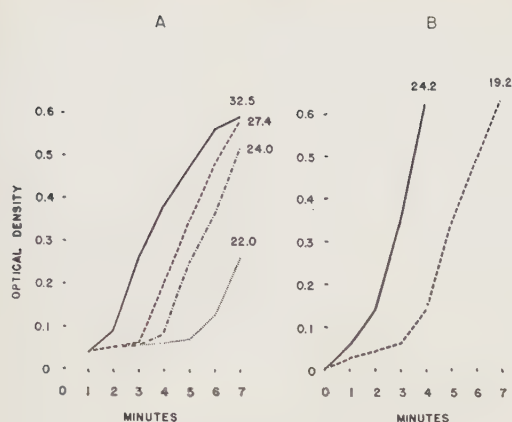


FIG. 1. Effect of temperature on Akerfeldt reaction. A—normal subject. B—psychotic patient.

velocity constant of the color-producing reaction and hence also the length of lag period which preceded it. Fig. 1A and 1B show the curves obtained with Akerfeldt's technic on sera drawn from 1 normal and 1 psychotic subject. The temperatures given are initial temperatures of the reaction mixture. Some curves also were obtained at temperatures other than 23°C. It is clear that at temperatures above 27°C all sera tested gave "psychotic" curves (*i.e.*, caused rapid onset of color) and that at temperatures below 17°C all sera tested were "normal" (*i.e.*, showed slow onset of color).

2) *pH*: The color-producing reaction is strongly inhibited at pH's above 7 and below 6. Variations in serum pH and buffer capacity have caused "psychotic" reactors to appear "normal" when an external buffer was not added to the reaction mixture (Fig. 2).

3) *Diet of the subjects*: Akerfeldt suggested that 3 distinct steps are involved in this reaction: 1. The DPP is oxidized linearly by blood ceruloplasmin, to a colored semi-quinone, DPPox. 2. This DPPox is immediately reduced to DPP by ascorbic acid of the serum. 3. After the ascorbic acid is used up, DPPox can re-form, imparting color to the reaction mixture. Later the DPPox reacts with other endogenous inhibitors in the serum—which perhaps includes a protein-containing sulphhydryl group—and thus the linearity of the first-order reaction is no longer apparent.

If the ascorbic acid is responsible for the lag period seen in this reaction, it would be reasonable to expect that differences in ascorbic-acid intake should cause variations in time that elapses before color-formation begins. It therefore becomes important to learn whether variation in ingested ascorbic acid might cause a positive test for psychosis in persons merely suffering from malnutrition. The method was first standardized with respect to temperature and pH. In this procedure 2 ml of fresh serum is mixed with 1 ml of 0.1 M phosphate buffer at pH 6.8 and ½ ml of distilled water, and the mixture cooled to 23°C. To this is added ½ ml of 0.4% DPP dissolved in 0.024 N HCl at 23°C (to yield DPP • 2HCl in solution). This mixture serves as its own blank in a Coleman spectrophotometer. The optical density of the mixture is measured at one-minute intervals at 552 μ ; between readings it is kept at 23° in a water bath. Several minutes after the rate of the color-producing reaction has started to decrease, or about 10 to 15 minutes after the DPP is added, the readings are terminated and the pH of the mixture is determined. This should be in the range of 6.8 ± 0.2 and is generally over 6.8.

Serum-oxidase tests were carried out before and after ingestion of the ascorbic acid on 5 chronically psychotic patients at the McLean

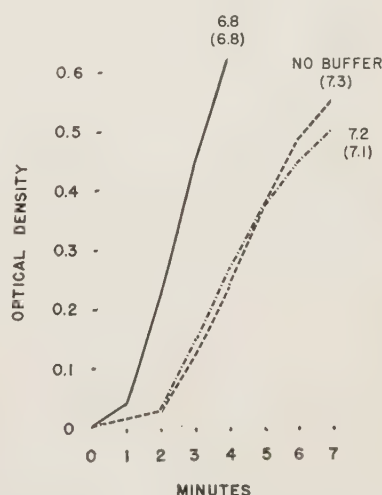


FIG. 2. Effect of pH on Akerfeldt reaction of psychotic patient. Figures indicate pH of buffer, where used; final pH of reaction mixture is in parentheses.

Hospital and on 9 healthy normal subjects. The first group, comprising 4 normal subjects, was given 48 oz. of orange juice (in addition to their normal diets), according to the following schedule:

| | |
|---------------------|---------|
| 1st test | 10 A.M. |
| Orange juice, 12 oz | 2 P.M. |
| <i>Idem</i> | 8 P.M. |
| Orange juice, 24 oz | 7 A.M. |
| 2nd test | 10 A.M. |

A second group, consisting of 5 psychotic patients, was given 1000 mg of the ascorbic acid, 250 mg each at 2 p.m. and 8 p.m., and 500 mg at 7 a.m. the next day, to demonstrate that the ascorbic acid actually was the agent responsible for the change in lag-time. A

third group of 5 normal subjects was similarly given 1000 mg of ascorbic acid over a 24-hour period.

There was an increase in lag-time after ascorbic-acid ingestion in every case (Fig. 3). In the first group, the average increase was 1.9 minutes; in the second 2.7 minutes; and in the third group it was 4.9 minutes.

The findings are consistent with the reaction hypothesized by Akerfeldt. They also indicate that the amount of ascorbic acid ingested in the period preceding the test is probably the most important determinant of the lag-time. The difference in slope that was found after the color-forming reaction began did not show any consistent relation to ascorbic-acid ingestion. The increase in lag-time in normal subjects was found to be greater than that observed in psychotic patients in the small series reported here. It is evident that the test chiefly measures the state of nutrition with respect to vitamin C, and that the results should be abnormal in any condition in which vitamin C stores are depleted.

Summary and conclusions. The Akerfeldt test for serum-oxidase activity, originally proposed as a test for mental disease, should not be used for this purpose. Sera yield "normal" or "abnormal" results depending on temperature and pH at which the reaction is carried out. Even when the method is standardized to eliminate these two variables, the results obtained depend so greatly on the subjects' previous diet that they render the test of questionable value in the diagnosis of anything, except perhaps ascorbic-acid deficiency.

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Received December 20, 1957. P.S.E.B.M., 1958, v97.

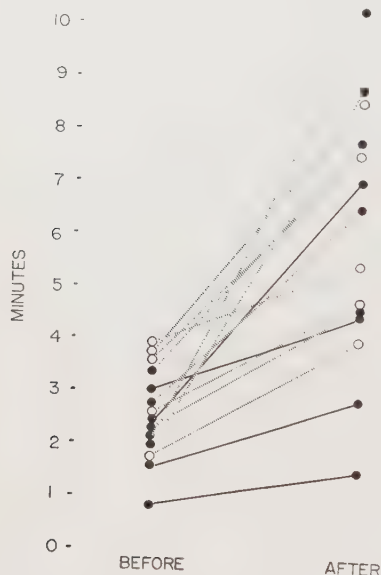


FIG. 3. Effect of ingestion of orange juice or of ascorbic acid on appearance time of color. Open circles indicate patients; solid circles indicate normal subjects. Solid lines indicate use of orange juice; dotted lines indicate use of ascorbic acid.

Inhibition of Thromboplastin Generation by Hyaluronidase Preparations And Reversal by Lyophilized Platelet Material and Derivatives.* (23781)

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Hyaluronidase has been reported to inhibit blood coagulation in the absence of platelets (1). The depolymerizing effect of hyaluronidase on hyaluronic acid is only slightly reversed by platelets alone, but the combination of platelets and complement or Serum Prothrombin Conversion Accelerator (SPCA) have been shown to inhibit this activity of hyaluronidase as determined by viscosimetric methods(2).

The nature of the inhibitory effects of hyaluronidase on the coagulation mechanism and their relation to platelets, is not known. Studies on the effects of hyaluronidase on the generation of thromboplastin and on the reversal of these effects by platelet material and analogous components from other tissues are therefore reported.

Methods and materials. Bovine testicular hyaluronidase[†] was suspended in physiological saline or imidazole buffer (pH 7.4). Concentrations were varied from 0.03 to 1.0 mg of hyaluronidase preparation per ml of solution. Fresh platelet concentrates were prepared by differential centrifugation and then washed 6 times in physiological saline. Lyophilized platelet material (LPM) was prepared as described previously(3). Lipid extracts of platelets and of beef brain, containing the thromboplastin-generating activity, were prepared as reported elsewhere(4). Defatted LPM, the residue obtained following extraction of LPM with alcohol-ether (3:1), was used after storage at -20° for periods up to 10 months. The thromboplastin generation test of Biggs and Douglas(5) was modified as indicated in the results.

Results. A thromboplastin-generating mix-

ture produces small amounts of thromboplastin even if platelets or analogous thromboplastin-generating components are not added. In the course of the generation of thromboplastin under these conditions, the clotting time of the substrate plasma decreases from over 2 minutes at the beginning of the incubation to between 35 and 50 seconds after incubating the mixture for more than 10 minutes (Fig. 1).

When hyaluronidase is added to such a platelet-deficient thromboplastin-generating mixture, the formation of thromboplastin is inhibited (Fig. 1).

The addition of fresh platelets or lyophilized platelet material to the hyaluronidase-containing mixture, reverses the inhibition of thromboplastin formation. (Fig. 1). Lipid extracts of lyophilized platelets or of beef brain have similar effects (Fig. 2).

The addition of defatted LPM (free of thromboplastin-generating activity) to a platelet-free thromboplastin-generating mixture containing hyaluronidase, returns the thromboplastin generation to control levels (Fig. 2).

An incomplete thromboplastin generation

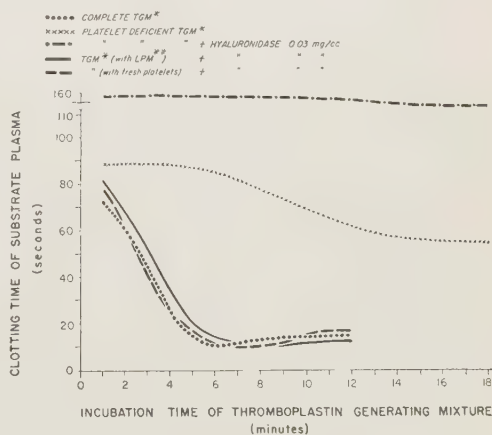


FIG. 1.

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[†] Worthington Biochemical Sales Co., Freehold, N. J.

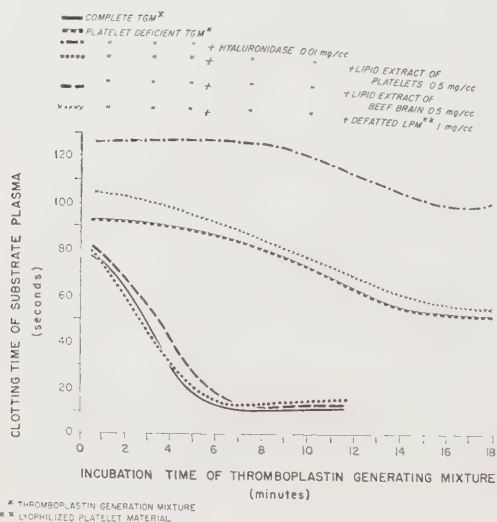


FIG. 2.

mixture, inhibited with hyaluronidase, was incubated for 9 minutes without evidence of thromboplastin formation. LPM was then added and normal thromboplastin formation occurred (Fig. 3). This indicates that hyaluronidase does not destroy an essential component of the coagulation mechanism, but rather acts as a reversible inhibitor.

Discussion. The data presented indicate that bovine hyaluronidase preparations inhibit the generation of plasma thromboplastin in the absence of adequate amounts of platelet material or its equivalent. The generation of thromboplastin after prolonged incubation of a mixture containing BaSO_4 -treated plasma, serum, calcium ions and buffer (instead of platelets or their equivalent) is probably due to traces of the lipid components of platelets which remain in the serum or the plasma after adsorption with BaSO_4 and subsequent high speed centrifugation. Such a system (incomplete thromboplastin-generation mixture) has been found suitable for studies of reactions in which the concentration of one or more platelet or plasma components may be critical.

Since, in our experience, hyaluronic acid and the products of its enzymatic degradation failed to produce significant effects on the generation of thromboplastin, the inhibition reported here may be due to an effect of hyaluronidase unrelated to its depolymerizing ac-

tivity. This is further suggested by the finding that thromboplastin generation is initiated when platelet material or its equivalent is added to an incomplete thromboplastin-generating system containing hyaluronidase which had been incubated for a period sufficient for the generation of thromboplastin. It should be emphasized that the hyaluronidase preparations used here were relatively impure and the inhibition of thromboplastin generation may have been due to contaminants. On the other hand, the effects described here were common to hyaluronidase preparations from a number of different sources.

In the presence of whole platelets (fresh or lyophilized), as well as of lipid extracts from platelets or brain, the thromboplastin generation reaches the usual levels which are observed in complete thromboplastin generation mixtures. This may be the result of either the direct inactivation of the inhibitory principle in the hyaluronidase preparation, or of compensatory effects of adequate concentrations of thromboplastin-generating agents.

The reversal of the effects of hyaluronidase in the incomplete thromboplastin generation mixture produced by defatted platelet material, free of demonstrable thromboplastin-generating component, suggests a direct mechanism of action. The specific action of hyaluronidase, however, can be inhibited by a large number of agents of biological origin and synthetic compounds(6). A nonspecific interaction of hyaluronidase with some of the nonlipid components of platelets could, therefore, account for the reversal of the effects of

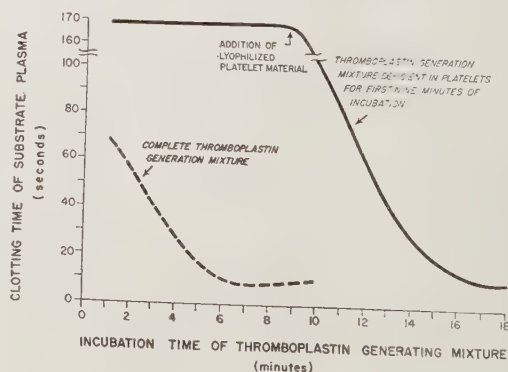


FIG. 3.

hyaluronidase on thromboplastin generation by defatted platelet material.

Although there is no indication that the apparent interactions between hyaluronidase and platelets play any role *in vivo*, further studies in regard to a possible relation of this phenomenon to vascular physiology and pathology may be warranted.

Summary. Bovine testicular hyaluronidase preparations inhibited formation of thromboplastin in absence of platelets or equivalent materials. This effect was not due to destruction of an essential component of the coagulation mechanism. Inhibition was prevented or reversed by platelets, thromboplastin-gen-

erating components extracted from platelets and tissues and by defatted platelet material.

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A New Group of Psychotomimetic Agents.* (23782)

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During the past few years, much interest has developed in psychotomimetic agents, particularly with regard to LSD 25 and mescaline. At the same time, considerable emphasis has been placed on the possible role of adrenalin and serotonin in psychoses, particularly because they are structurally related to the psychotomimetic agents and are pharmacologically antagonistic. The role of acetylcholine and acetylcholine-like substances, on the other hand, has received relatively little attention.

Knowledge of the hallucinogenic properties of cholinergic blocking agents, such as atropine and hyoscine, dates back to the time of the ancient Hindus. Recently, a group of piperidyl benzilates possessing anticholinergic properties were synthesized by Biel and associates(1) as possible antispasmodics in the treatment of duodenal ulcer(2). In the course of therapeutic trials, it was found that the tertiary amine hydrochlorides of the benzilate esters, although active anticholinergics, produced undesirable side effects, particu-

larly hallucinations. The quaternary ammonium salts, on the other hand, were entirely devoid of such effects. We have recently obtained a series of such substances and examined their psychotomimetic effects on animals and human subjects(3).

Methods. The psychotogenic effects of the N-methyl-3-piperidyl benzilate and related congeners were tested on over 40 human volunteers who were either normal or patients complaining of minor disorders. Although some of the patients had limited knowledge of the psychotogenic action of the drugs, the majority of subjects were completely unaware of their nature. Ceruloplasmin determinations were made on many subjects, employing a method described previously(4). All of the agents were tested for their behavioral effects in animals, including some 30 Siamese fighting fish, 50 rodents, and 5 cats. The action of these agents on the Siamese fighting fish is comparable to those described for LSD by Abramson(5). In rodents there were marked behavioral changes, such as initial excitement and marked hyperactivity, spontaneous squealing, lack of responsiveness to stimuli, muscular weakness, and lethargy.

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The anticholinergic effect of the agents was determined on isolated smooth muscle preparations and the rectus abdominus according to the method of Chang and Gaddum(6).

Results. Experimental findings have indicated that the compounds are extremely powerful hallucinogens, in many respects more interesting than LSD and mescaline. When administered in 5-15 mg doses, orally, to human volunteers, distinct auditory and visual hallucinations occurred within one hour in every individual and recurred periodically for periods up to 10 hours after administration of the drug. Hallucinations were accompanied by gross distortions of visual images and severe alterations in feeling state. A number of subjects exhibited paranoid and megalomaniac delusions, while the affective states ranged from a feeling of unpleasantness to extreme terror. Some of the subjects actually carried on conversations with imaginary individuals involving situations dating back 10-20 years. The following are almost exact quotations from different subjects: "People from India are standing outside a tent. They have turbans and those are camels." "I see six people sitting around a table playing cards . . . a monkey is over the table hanging by his tail." "I am walking down a narrow corridor and suddenly stop and cannot move . . . a band is playing . . . a drum is beating 3/4 rhythm."

The subjects receiving 10 mg (orally) of N-methyl-3-piperidyl benzilate were in complete loss of contact with the environment for many hours while experiencing dramatic visual and auditory hallucinations. In many respects these anticholinergic agents come closer to simulating clinical psychoses than do mescaline and LSD.

Thus far, a number of congeners have been tested for both hallucinogenic properties and anticholinergic effect on the isolated colon (Table I). Of all the compounds tested for hallucinogenic properties, N-methyl-3-piperidyl benzilate is the most potent, with the N-ethyl derivative being somewhat less effective. The tetramethyl derivative is considerably less effective than the N-ethyl derivative. The quaternary derivative is devoid of

psychotogenic effects. As for the antispasmodic potency, although the 3 substances possessing psychotogenic properties are perhaps the most potent, the remaining compounds are still quite effective.

Ceruloplasmin determinations were made on all subjects, since this enzyme was shown to be increased in the serum of acute schizophrenics(4,7). The method used has been described previously(4). Preliminary observations have indicated that as much as a 50-75% elevation in the blood ceruloplasmin accompanies the hallucinatory episode produced by these agents. The enzyme increased only when marked psychogenic disturbances were apparent, returning to normal shortly after the psychogenic effects disappeared and while peripheral autonomic effects, such as mydriasis, muscular weakness, and dryness of the mouth, still persisted. A rise in ceruloplasmin has been shown to accompany changes in affective or feeling states, regardless of the mechanism by which the effects are produced(3).

Discussion. A discussion of the relative antispasmodic properties of this group of compounds appears elsewhere(1). It is apparent from the present study that in this series of compounds there is no direct relationship between the anticholinergic effect on smooth muscle and psychotogenic potency. The presence of the hydroxyl group in the acid moiety to yield the diphenylacetate ester is undoubtedly essential for hallucinogenic effect, while only slightly enhancing the anticholinergic effect. Since both the diphenylacetate and the benzilate derivatives penetrate the blood brain barrier, it would appear that the hydroxyl group is an absolute requirement. The presence of a quaternary nitrogen in the piperidine ring only slightly influences the anticholinergic effect, but apparently prevents the compound from penetrating the blood-brain barrier. As a rule, quaternary ammonium compounds are not able to enter the central nervous system through the blood stream. Preliminary observations have shown that intrathecal injections of the quaternary compound into rats produce much the same kind of neurological and behavioral disturb-

TABLE I. Structure-Activity Relationships of Some Piperidyl Benzilate Congeners. Anticholinergic effect was determined on isolated rat colon with concentrations of about 10^{-6} M.

| $\begin{array}{c} \text{O} \quad \text{X} \\ \parallel \quad \\ \text{R}-\text{O}-\text{C}-\text{C} \begin{array}{l} \diagup \text{C}_6\text{H}_5 \\ \diagdown \text{C}_6\text{H}_5 \end{array} \end{array}$ | | | | |
|--|---|----|---------------------------------|----------------------------------|
| Name | R | X | Relative hallucinogenic potency | Relative anticholinergic potency |
| N-methyl-3-piperidyl-benzilate | | OH | ++++ | ++++ |
| N-ethyl-3-piperidyl-benzilate | | OH | +++ | +++ |
| 1,2,2,6 tetramethyl-4-piperidyl benzilate | | OH | — + | +++ |
| N-ethyl-3-piperidyl-diphenylacetate | | H | 0 | +++ |
| N-dimethyl-3-piperidyl benzilate | | OH | 0 | +++ |

ances observed with the tertiary benzilates. At present, numerous other congeners are being examined for their hallucinogenic properties. Future synthetic work is contemplated in an effort to explore other structure-activity relationships from the point of view of hallucinogenic effect. In view of the work of others on anticholinergic substances, it may be predicted that the distance between the hydroxyl group and the piperidyl nitrogen is critical(8,9). Introduction of alkyl groups into the molecule would, therefore, presumably diminish the anticholinergic potency, and it will be of interest to determine the relationship of such a change to hallucinogenic effectiveness.

Summary. A series of synthetic anticholin-

ergic agents have been shown to possess potent psychotomimetic properties. Chemically, the agents are esters of piperidine and benzoic acid. Among the effects produced are megalomaniac and paranoid delusions, visual and auditory hallucinations, and a partial loss of contact with the environment. A number of congeners of the compounds have been examined with regard to structure-activity relationships.

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